Isolation and characterisation of an evolutionary conserved protein that is involved in photosystem I biogenesis in *Arabidopsis thaliana*

Doctoral dissertation

submitted by

Paul-Hendrik Hein

Date of Birth: 29th April 1979

Place of birth: Halle

Faculty of Biology and Pharmaceutics
Institute of General Botany and Plant Physiology
Friedrich-Schiller-University Jena

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Gutachter:

1. Prof. Dr. Ralf Oelmüller
2. PD Dr. Thomas Pfannschmidt
3. Prof. Dr. Daguang Cai

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Abbreviations

General abbreviations in common use, chemicals and enzymes

ATP	Adenosine triphosphate
BSA	Albumine Bovine Serum
CAPS	Cleaved amplified polymorphic sequences
cDNA	Complementary deoxyribose nucleic acid
Chl	Chlorophyll
Col	*Columbia*
CTP	Cytosine triphosphate
DNA	Deoxyribose nucleic acid
DNase	Deoxyribonuclease
dNTP	Desoxynucleoside triphosphate
EDTA	Ethylenediaminetetraacetate
EMS	Ethyl methanesulfonate
GTP	Guanosine triphosphate
HCl	Hydrochloric acid
HEPES	N-2-Hydroxyethylpiperazin-N`-2-ethanolsulfonic acid
Ler	*Landsberg erecta*
MOPS	3-N-morpholino-propanesulfonic acid
mRNA	Messenger ribonucleic acid
NaOH	Sodium hydroxide
NTP	Nucleoside triphosphate
PAGE	Polyacrylamide gel electrophoresis
PVDF	Polyvinylidene-difluoride
RNA	Ribonucleic acid
RNase	Ribonuclease

SDS	Sodiumdodecylsulfate
SSLP	Simple sequence length polymorphisms
Tris	Tris-(hydroxymethyl)-aminomethan
UTP	Uridine triphosphate

Measuring units

bp	Base pairs
°C	Degree Celsius
Ci	Curie
kb	Kilo base pairs
kDa	Kilodalton
l	Litre
M	Molar concentration
min	Minute
rpm	Rotations per minute
u	Enzymatic unit
v/v	Volume per volume
w/v	Weight per volume
w/w	Weight per weight

I. Introduction

1.1. Photosystem I complex

The photosystem I (PSI) is a pigment-protein complex located in the thylakoid membranes of cyanobacteria and chloroplasts of algae and higher plants, which functions as a plastocyanin (or cytochrome c_6)-ferredoxin oxidoreductase (Chitnis 2001; Jensen et al., 2007). The complex consists of a reaction centre core and an associated light-harvesting antenna complex (LHC) which is composed of chlorophylls, carotenoids and chlorophyll *a*/*b*-binding proteins (LHCPs) required for capturing most of the light energy. Genes for six PSI-LHCPs have been identified in *Arabidopsis* (cf. Jansson 1999 and references therein) and at least one copy of four LHCPs, Lhca1-4, is present in a PSI-LHCI complex (Ben-Shem et al., 2003; Ballottari et al., 2004). The presence of a fifth LHC polypeptide, Lhc5, in the PSI antenna has been reported by Ganeteg et al. (2004). Light is also captured by chlorophylls and β-carotenes associated with the reaction centre core (Jordan et al., 2001), which function as additional inner antenna. The antenna size of PSI varies depending on the light intensity and spectral distribution as well as on other environmental factors (Bailey et al., 2001).

The excitation energy captured by the pigments is delivered to a special chlorophyll *a*-pair, P_{700}, in the reaction centre (RC) of the core complex. P_{700} is responsible for charge-separation, which is followed by a series of redox reactions and ultimately the reduction of ferredoxin at the reducing site of PSI. The reducing potential of ferredoxin is utilized for a variety of biochemical processes, such as the reduction of $NADP^+$, or the assimilation of nitrate or sulfate (Ben-Shem et al., 2003; Nelson and Yocum, 2006).

In 2003, the first crystal structure of PSI from a higher plant was determined (Ben-Shem et al., 2003). In contrast to the trimeric cyanobacterial PSI (Jordan et al., 2001; Chitinis 2001), the plant PSI was purified as a monomer. At least 15 different polypeptide subunits (PSI-A-L and PSI-N-P) are required for the backbone of the PSI core of higher

plants (Chitnis 1996; Chitnis 2001; Jensen et al., 2004; Jensen et al., 2007). In eukaryotes, five of them, PSI-A, PSI-B, PSI-C, PSI-I and PSI-J are plastome-encoded, while the residual ones are encoded in the nucleus (Shinozaki et al., 1986; Hayashida et al., 1987; Sugiura 2003). Five subunits (PSI-G, -H, -N, -O and -P) have not been detected in cyanobacteria, while PSI-M is not present in the PSI of angiosperms (cf. below).

PSI-A and PSI-B, the two largest polypeptide subunits with 11 transmembrane helices each, form a heterodimer and bind the primary electron donor P_{700}, the electron acceptors A_0 (a chlorophyll *a* molecule), A_1 (a phylloquinone), F_X (a [4Fe-4S] cluster) and most of the remaining PSI-cofactors including chlorophyll *a* and ß-carotene molecules. The terminal two cofactors involved in the electron transfer, the two [4Fe-4S] clusters F_A and F_B, are bound by PSI-C at the reducing site of the complex (Chitnis 2001).

The initial step in PSI biogenesis is the formation of the heterodimer PSI-A/B. In *Chlamydomonas reinhardtii*, PSI-B seems to be required for stable PSI-A accumulation and translation of its message (cf. below). Subsequently, the presence of PSI-A is required for stable PSI-C accumulation and association with the PSI-A/B dimer (Wostrikoff et al., 2004).

1.2. Mutant analysis helped to elucidate the role of polypeptide subunits in PSI function

Besides the crystal structure, most of the information about the function of the PSI subunits derives from mutants impaired in one or more subunit genes or from biochemical studies. The importance of the PSI-A/B dimer for the assembly of the complex has been demonstrated for a variety of pro- and eukaryotic mutants. Mutants lacking PSI-A/B generally fail to assemble the entire core complex, although some of the more peripheral subunits can accumulate in the thylakoid membranes. In a

Synechocystis psaA mutant, generated by insertional mutagenesis (Smart et al., 1991), the *psaA*/*B* mRNA is truncated and the P_{700} apoproteins cannot be detected. Similarly, a *Synechocystis* strain lacking a functional *psaA*/*B* operon shows no accumulation of the PSI core, does not grow autotrophically and has dramatically reduced chlorophyll levels (Shen et al., 1993). This was also confirmed for the eukaryotic green alga *Chlamydomonas* (Cournac et al., 1997; Redding et al., 1999).

In eukaryotes, the three cofactor-binding proteins PsaA, -B and -C are encoded by plastid genes and their domain structures are highly conserved. PSI-A and PSI-B assemble at an early step of PSI biogenesis (Smart et al., 1993), forming the chlorophyll *a*-protein complex CPI, that binds most of the cofactors and pigments of PSI. PSI-B is required for significant PSI-A synthesis, in the absence of PSI-B unassembled PSI-A represses its own translation (Wostrikoff et al., 2004). CPI is the template for PSI-C binding. PSI-C, a 9-kDa protein located at the reducing site of the complex, coordinates the Fe-S clusters F_A and F_B through two cysteine-rich domains. Again, in the absence of CPI, unassembled PSI-C represses its own translation. This suggests that significant accumulation of PSI-A and -C occurs only when the previous assembly steps are completed. Thus, PSI-B accumulation is the main control step in the biogenesis of PSI in *Chlamydomonas* (Wostrikoff et al., 2004).

Studies on PSI-C revealed differences between pro- and eukaryotic PSI. While the prokaryotic PSI assembles properly in PSI-C deficient mutants and is functional (Mannan et al., 1991), the absence of this protein in eukaryotes leads to an unassembled PSI (Takahashi et al., 1991). This indicates that besides evolutionary conservation, the importance of this subunit for the electron transfer and the assembly process differs.

The other, more peripheral subunits bind to the assembled PSI-A, -B and -C core complex. *Arabidopsis* mutants lacking PSI-D do not grow photoautotrophically, have reduced levels of all other subunits and do not assemble a functional PSI complex

(Haldrup et al., 2003). PSI-D and -C provide a docking niche for ferredoxin. Furthermore, PSI-D is involved in the stable association of PSI-C, -E and -L into the PSI complex (Kruip et al., 1997; Xu et al., 2001). A transposon insertion line of *Arabidopsis* with disrupted *PsaE1* (Varotto et al., 2000) showed a light sensitive phenotype and had increased levels of chlorophyll fluorescence and photoinhibition. Besides its structural role on the reducing site of the complex, PSI-E is also involved in the cyclic electron flow. The role of PSI-F as plastocyanin docking site was shown for *Chlamydomonas* and *Arabidopsis* (Farah et al., 1995; Haldrup et al., 2000). Inactivation of *PsaF* in *Chlamydomonas* results in impaired electron transport from plastocyanin to PSI, although PSI still assembles and mutants grow photoautotrophically (Farah et al., 1995). In contrast, PSI-F in *Arabidopsis* is important for photoautotrophic growth. Plants lacking PSI-F are chronically photoinhibited and show disturbed energy transfer from Lhc1 to P_{700} (Haldrup et al., 2000). In cyanobacteria, PSI-F provides the docking site for cytochrome c_6.

PSI-G and -H are restricted to green algae and higher plants and *Arabidopsis* mutants lacking PSI-G or PSI-H grow like wild type plants under standard conditions (Naver et al., 1999; Jensen et al., 2002). Kjaerulff et al. (1993) suggest that PSI-G has the same ancestor as PSI-K. PSI-H is important for the adaptation of plants to different light conditions (Lunde et al., 2000). When plants are illuminated with light which is preferentially absorbed by either PSII or PSI, they redistribute excitation towards the light-limiting photosystem. If excitation of PSI is limited, plants phosphorylate and detach the mobile antenna LHCPII from PSII, which migrates to PSI. Lunde et al. (2000) have shown that LHCII cannot transfer energy to PSI in mutants lacking PSI-H. Thus, PSI-H probably forms the docking site for the mobile antenna. Furthermore, PSI-H hinders the formation of contacts among PSI monomers, so that trimer formation does not occur in higher plants (Ben-Shem et al., 2003). Trimerization was probably

lost in plants to facilitate re-allocation of phospohorylated LHCII to PSI under light conditions favouring PSII excitation (cf. Ben-Shem et al., 2003).

In contrast to PsaD-H, the two small subunits PSI-I and -J of 4-kDa and 5-kDa, respectively, are plastome-encoded. Whether translation of their messages is also downregulated when the translation products are not stabilized at the assembling PSI complex, is unknown at present. PSI-I is required for a proper assembly of PSI-L and inactivation of *psaI* in cyanobacterial mutants decreases the PSI-L protein levels to 80 % (Xu et al., 1995). In addition, deletion of the cyanobacterial PSI-J reduces both *PsaF* mRNA and the amount of PSI-F (Xu et al., 1994).

PSI-K is crucial for the interaction of the PSI core with the LHCI antenna. However, *Arabidopsis* mutants lacking PSI-K appear to grow like wild type plants under standardized conditions. In several cyanobacteria, more than one PSI-K is present. *Synechocystis* contains two PSI-K subunits, K1 and K2. PSI-K2 is involved in the transfer of excitation energy from the phycobilisomes to the reaction centre under high-light conditions. Thus, PSI-K2 is involved in high-light acclimation (Fujimori et al., 2005; cf. below).

PSI-L is thought to be responsible for trimerization of PSI in *Synechocystis* through the C-terminal part of the polypeptide which protrudes in the reaction centre (Chitnis et al., 1993; Ben-Shem et al., 2003). The C-terminal extension is missing in the plant PSI-L. Apparently, PSI-L in *Synechocystis* is not required for photoautotrophic growth and PSI assembly (Chitnis et al., 1993). *Arabidopsis* plants lacking PSI-L also have reduced levels of PSI-H (and PSI-O), and therefore PSI-L can be grouped into state transitions mutants (Lunde et al., 2000). PSI-M is a cyanobacterial subunit of PSI. *PsaM* genes are also present on the plastid genome of several algae and gymnosperms, but have not been found in angiosperms. In *Synechocystis* lacking PSI-M, trimerization of PSI is

impaired, although photoautotrophic growth and photosynthetic activity appear to be unaffected (Naithani et al., 2000).

PSI-N is only present in higher plants. It is involved in docking of plastocyanin to PSI. Mutant analysis in *Arabidopsis* revealed impaired electron transfer from plastocyanin to P_{700}^{+} (Haldrup et al., 1999). However, absence of PSI-N does not affect PSI assembly and photoautotrophic growth in *Arabidopsis* (Haldrup et al., 1999).

Recently, two novel PSI subunits, PSI-O (Knoetzel et al., 2002; Jensen et al., 2004) and PSI-P (Khrouchtchova et al., 2005), have been described. PSI-O is restricted to eukaryotes. Mutant analysis in *Arabidopsis* revealed that PSI-O requires the presence of PSI-H and PSI-L, and together with them, it might form a structure within PSI being responsible for state transition (Jensen et al., 2004). The exact role of PSI-P is still unknown.

1.3. Regulatory proteins

Only a few regulatory proteins controlling the expression of the plastid-encoded genes, translation of their messages on plastid ribosomes, the assembly of the complex with the proper association of all cofactors and the integration of the complex into the thylakoid membranes have been identified. The correct assembly of the pigment-protein-complex in eukaryotes requires the interplay between nucleus and chloroplasts. Genes for the nuclear-encoded subunits including most of the regulatory proteins are transcribed and the messages translated on cytosolic ribosomes. The synthesized precursor polypeptides in the cytosol are recognized by the plastid import machinery at the surface of the organelle and subsequently imported into plastids, cleaved to the mature protein and targeted to their correct location. Transcription and translation of the plastid-encoded proteins occur by the expression machinery located in the plastids, thus formation of the

PSI complex must be highly regulated and coordinated between nucleus and plastids (Barkan and Goldschmidt-Clermont, 2000).

Mutants which are unable to perform photosynthesis can be isolated easily by their inability to grow photoautotrophically, or by a high chlorophyll fluorescence phenotype, or both. If the light energy, captured by the photosynthetic pigments, cannot be utilized for photochemical reactions, it is emitted from the pigments as fluorescence. That can be detected as red light under UV irradiation. Among the photosynthesis mutants, PSI affected plants can be selected by the lack of the P_{700} redox function. Many of these mutants have been isolated from *Synechocystis*, *Synechococcus*, *Chlamydomonas*, *Arabidopsis* or maize, to mention a few well studied model systems for PSI research. Here some of the regulatory factors identified and characterized in those mutants are described

1.3.1. Synthesis of the Fe-S cluster

Fe-S clusters are believed to be among the oldest structures found in living organisms. PSI contains three 4[Fe-S] clusters and the electron acceptor ferredoxin one 2[Fe-S] cluster. Biochemical and genetic studies suggest that chloroplasts have their own Fe-S biosynthesis machinery. However, little is known about the enzymes involved in the biogenesis and the assembly of the cluster into their target protein (complexes). Cystein is the sulphur source and formation of the cluster involves a plastid-localized NifS protein (Pilon-Smits et al., 2002; Leon et al., 2002). At least ten genes have been identified which might have functions in Fe-S cluster biogenesis in *Arabidopsis*, however, their roles is mainly unknown.

In *Synechococcus*, inactivation of *rubA* revealed a novel PSI mutant (Shen et al., 2002a), which does not grow photoautotrophically and lacks PSI activity, although all membrane intrinsic PSI subunits are present. The three iron-sulfur clusters, F_X, F_A and

F_B, are not present in the monomeric PSI complexes and the loss of F_A and F_B might be a secondary effect due to the absence of F_X (Shen et al., 2002b). Based on these observations, it has been proposed that RubA is required for the assembly of the F_X Fe-S cluster. The protein is present in purified thylakoid membranes of *Synechocystis*, but not in the plasma membrane preparations (Shen et al., 2002a). *RubA* homologs are found in cyanobacteria, prochlorophytes, cryptomonads, green algae and higher plants, and thus, most likely in all oxygen-evolving photosynthetic organisms. The presence of RubA in spinach and *Chlamydomonas* was confirmed immunologically (Shen et al., 2002 a).

AtCnfU-V and AtCnfU-IVb are two recently described chloroplast-localized NifU-like proteins in *Arabidopsis* (Yabe et al., 2004). *Arabidopsis atCnfU-V* mutants have a pale-green and dwarf phenotype, reduced amounts of PSI and ferredoxin, as well as lower activity of stromal Fe-S cluster insertion activity (Yabe et al., 2004). AtCnfU is proposed to function as a scaffold protein for Fe-S clustering in the chloroplast and thus, is required for biogenesis of ferredoxin and PSI (Yabe et al., 2004).

hcf101 is a novel mutant impaired in PSI biogenesis (Lezhneva et al., 2004; Stöckel and Oelmüller, 2004). The gene *Hcf101* encodes a MRP-like protein with a nucleotide-binding domain. PSI subunits are synthesized in *Arabidopsis* mutants lacking Hcf101 but do not assemble into a stable complex. Hcf101 is a soluble protein and only loosely associated with membranes. *hcf101* mutants have lesions in Fe-S cluster biogenesis, although the exact function of the protein is unknown at present. Hcf101 could be involved in delivering components from the stroma to the assembly site of PSI in the stroma thylakoids. *Synechocystis* mutants with a deletion in the *Hcf101* homolog, *Slr0067*, still grow photoautotrophically, but are severely impaired in their Fe/S metabolism (Stöckel and Oelmüller; submitted). Thus, this couple of regulatory proteins provides an interesting system to study homologous genes and their function in prokaryotes and eukaryotes. Comparison of the roles of PSI-C (and other structural

components), *ycf4*, *Hcf101*/*slr0067* and *Pyg7*/*ycf37* for cyanobacteria and eukaryotic PSI clearly demonstrates that deletion of any of these components in cyanobacterial causes an impairment in PSI accumulation or function, while comparable mutants in plants are unable to assemble the entire complex.

The regulatory protein "accumulation of photosystem I" (APO1) is specifically required for [4Fe-4S] cluster assembly in chloroplasts of *Arabidopsis* (Amann et al., 2004). *apo1* mutants do not grow photoautotrophically and PSI core subunits are barely detectable (Amann et al., 2004). Several [4Fe-4S] cluster-containing complexes in plastids, such as the ferredoxin-thioredoxin reductase and the NADP(H)-dependent dehydrogenase are also missing in the mutant, while the [2Fe-2S] cluster containing ferredoxin is present. Thus, based on a 100 amino acid containing motif, APO1 is thought to provide ligands for [4Fe-4S] clusters (Amann et al., 2004). Four APO1 homologs with unknown function are present in the *Arabidopsis* genome and similar genes are present only in vascular plants. Because of the different phenotypes of *hcf101* and *apo1* mutants, with *apo1* mutants showing a stronger phenotype, both proteins might be required for different steps in the Fe-S cluster biogenesis in plastids.

1.3.2. Factors involved in translation of PSI messages

Two proteins, translation of *psaB* (TAB)1 and TAB2, are required for *psaB* translation in *Chlamydomonas*. In both mutants no PSI-A and PSI-B accumulation can be detected (Stampacchia et al., 1997). Transgenic *Chlamydomonas* cells with the 5´-UTR of *psaB*, fused to a resistance gene, demonstrated that the reporter gene is not expressed in the TAB1 and TAB2 mutant background. This indicates that the target sites for TAB1 and TAB2 are located in the 5´-UTR of *psaB* and thus, these proteins are most likely required for translation initiation of this mRNA (Dauvillee et al., 2003). Interestingly, polyribosome-loading of the *psaB* message was severely reduced in the TAB2 mutant, while the *psaA* message was not reduced. This indicates that the downregulated protein

levels of PSI-A and PSI-B are caused by different mechanisms: PSI-B is reduced because of the lower translation rate of its message, while PSI-A is most likely reduced, because the synthesized protein is degraded in the absence of PSI-B (Rochaix et al., 2004). While no obvious homologous counterpart could be identified for TAB1 in other species, TAB2 is conserved in photosynthetic organisms including *Arabidopsis*.

Recently, an *Arabidopsis* knockout line of the homologous *Chlamydomonas* Tab2, Atab2, was described and Atab2 was found to be a RNA binding protein that might activate photosystem protein translation (Barneche et al., 2006).

1.3.3. Protein accumulation and/or assembly mutants

Targeted inactivation of *ycf3*, a hypothetical chloroplast open reading frame (ORF) in higher plants, uncovered a gene involved in assembly of PSI (Ruf et al., 1997). Plants lacking Ycf3 specifically lack PSI subunits, whereas transcripts of plastid encoded PSI genes accumulate to wild type levels. The same observations were made in *Chlamydomonas* mutants lacking either Ycf3 or Ycf4. Both proteins are necessary for the accumulation of the entire complex (Boudreau et al., 1997). Later on, it was shown that Ycf3 specifically interacts with PSI-A and PSI-D (Naver et al., 2001). Ycf4 is associated with a high-molecular mass complex which contains also subunits of PSI (Rochaix et al., 2004). Inactivation of *Ycf4* in *Synechocystis* still allows PSI accumulation, although to a lower extent (Wilde et al., 1995; cf. below).

Inactivation of the *Synechocystis* ORF *slr0171* decreases the PSI/PSII ratio, and Slr0171 is further suggested to be an assembly or stability factor of PSI (Wilde et al., 2001). This ORF shows similarity to the conserved chloroplast ORF *ycf37* of several algae and encodes a TPR protein (Wilde et al., 2001). More recently, Dühring et al. (2007) have shown that Ycf37 is involved in late assembly steps in the cyanobacterial PSI. The authors propose that a PSI-L/PSI-K less monomeric PSI complex, called PSI**, is

formed first. Integration of PSI-L results in the formation of the PSI* intermediate. These two monomeric complexes are missing in the *ycf37* mutant, indicating that Ycf37 is either required for the synthesis or the stabilization of the intermediates. Finally, integration of one of the two PSI-K subunits leads to the formation of the complete PSI monomer in *Synechocystis*, prior to trimerization. PSI-K2 is assembled into PSI* at high-light intensities, while PSI-K1 is assembled under normal light conditions (Dühring et al., 2007). The presence of PSI* intermediates, in which different PSI-K subunits can be integrated, allows a rapid re-adaptation of PSI to different light conditions. In *Arabidopsis,* the homologous gene, named *Pale yellow green7* (*Pyg7*), is encoded in the nucleus, suggesting a gene transfer during evolution. The *pyg7* mutant is deficient in PSI. PSI subunits are synthesized, but do not assemble into a stable complex. The mutant fails to grow autotrophically and shows alterations in leaf coloration and plastid ultrastructure. *Pyg7* was isolated by map-based cloning and encodes a TPR protein (Stöckel et al., 2006). Immunological studies with antibodies against Pyg7 revealed that the protein is present in thylakoid membrane fractions of *Arabidopsis* leaf extracts. In addition, when analyzed in sucrose gradients, Pyg7 co-purifies with PSI. While cyanobacteria can still grow photoautotrophically in the absence of functional Ycf37 (Wilde et al., 2001), Pyg7 is absolutely necessary for photoautotrophic growth and proper PSI function. To this end, *ycf37* and *Pyg7* provide an interesting system to compare genes and their function in prokaryotes and eukaryotes.

The *cyanobacterial* BtpA protein was found to stabilise the PSI reaction centre (Bartsevich and Pakrasi 1997; Zak et al., 1999; Zak and Pakrasi 2000). BtpA is associated with thylakoid membranes and required for stability of PsaA and the PSI reaction centre, respectively, at low temperature.

Many photosynthetic mutants with nuclear lesions were also isolated from maize (Barkan et al., 1986; Heck et al., 1999). *hcf44*, for instance, was isolated from a variety

of ethyl methanesulfonate-induced (EMS) mutants (Heck et al., 1999). PSI-C, PSI-D and PSI-E subunits are missing in *hcf44* mutants, while PSI-A/B accumulation was not impaired (Heck et al., 1999). Since PSI-C is required for the assembly of PSI-D and PSI-E in *Synechocystis* (Yu et al., 1995), it is reasonable to assume that either PSI-C synthesis or PSI-C integration into the PSI complex is defective in *hcf44* mutants (Heck et al., 1999).

1.3.4. *Trans*-splicing mutants from *Chlamydomonas*

About one fourth of the PSI mutants, which were isolated from *Chlamydomonas,* are specifically affected in the splicing of the *psaA* precursor transcripts (Goldschmidt-Clermont et al., 1990). The maturation of the *Chlamydomonas psaA* transcript requires two *trans*-splicing steps. Raa1 is required for *trans*-splicing of both *psaA* introns (Merendino et al., 2006). Raa2 is involved in *trans*-splicing of the second *psaA* intron only (Perron et al., 1999). Interestingly, Raa2 shares sequence similarity to pseudouridine synthases, however, exchange of several amino acid residues essential for the pseudouridine synthase activity did not alter-*trans* splicing. This indicates that this enzyme activity is not required for *trans*-splicing (Perron et al., 1999; Rochaix et al., 2004). Both proteins are membrane-associated. In contrast, Raa3 is a soluble protein and part of a high-molecular weight complex. Raa3 is required for *trans*-splicing of the first intron of *psaA* (Rivier et al., 2001). It has been proposed that the splicing of the first intron occurs first in the chloroplast stroma, followed by splicing reactions at the thylakoid membrane (Rochaix et al., 2004)

1.4. Purpose of the present project

The regulatory mechanisms during early events in PSI biogenesis are still enigmatic. As the formation of the PSI-A/B heterodimer is believed to be the initial step of PSI biogenesis, it is interesting to understand the principles underlying the regulation of the

psaA/*B* gene expression. In this work, *Arabidopis* was chosen as model organism, because a vast amount of sequence information of several ecotypes (e.g. *Landsberg erecta*; Lukowitz et al., 2000) is available. The mutagen ethyl methanesulfonate (EMS) was used to produce mutants that are impaired in PSI biogenesis, with the aim to identify novel regulatory factors for PSI biogenesis *via* map-based cloning. Thus, a recessive *Arabidopsis* mutant, *hcfb12-1* (high chlorophyll fluorescence), that fails to accumulate proper *psaA*/*B* transcript levels, was isolated from an EMS mutagenized *Arabidopsis* seed pool.

The purpose of this work was to characterise the mutant at the molecular and physiological level, and to identify the mutated locus *via* positional cloning. Among these characterisation of *hcfb12-1*, it was further of particular importance to characterize the identified protein in context of PSI biogenesis, and to elucidate the primary reason of the lower *psaA*/*B* transcript abundance in *hcfb12-1*.

II. Materials and Methods

2.1. Materials

2.1.1. Chemicals

Basic chemicals were obtained from:

- Amersham Biosciences Europe GmbH, Freiburg
- Carl Roth GmbH, Karlsruhe
- Duchefa Biochemie B.V., Haarlem, Netherlands
- Life Technologies GmbH, Karlsruhe
- Merck Biosciences GmbH, Schwalbach
- SERVA Electrophoresis GmbH, Heidelberg
- Sigma-Aldrich Chemie GmbH, Taufkirchen

2.1.2. Enzymes and kits

Enzymes:

• DNase	MBI Fermentas GmbH, St. Leon-Rot
• M-MuLV reverse transcriptase	MBI Fermentas GmbH, St. Leon-Rot
• *Pfu* DNA polymerase	Promega GmbH, Mannheim
• Restriction endonucleases	MBI Fermentas GmbH, St. Leon-Rot
• RNase	MBI Fermentas GmbH, St. Leon-Rot
• *Taq* DNA polymerases	MBI Fermentas GmbH, St. Leon-Rot
• T4 DNA ligase	Promega GmbH, Mannheim

Kits:

• CycleReader^{TM} DNA sequencing kit	MBI Fermentas GmbH, St. Leon-Rot

- pGEM®-T Vector system — Promega GmbH, Mannheim
- Qiaex II gel extraction kit — Qiagen GmbH, Hilden
- Random prime labelling system — Amersham Biosciences Europe GmbH, Freiburg
- RevertAid™ first strand cDNA Synthesis kit — MBI Fermentas GmbH, St. Leon-Rot

2.1.3. DNA markers for electrophoresis

- GeneRuler™ DNA ladder — MBI Fermentas GmbH, St. Leon-Rot
- λ DNA/*Eco*RI+*Hind*III — MBI Fermentas GmbH, St. Leon-Rot

2.1.4. Nucleotides and isotopes

- dNTP set — MBI Fermentas GmbH, St. Leon-Rot
- NTP set — MBI Fermentas GmbH, St. Leon-Rot
- α-^{32}P dCTP (10 TBq/mmol) — Hartmann analytic GmbH, Braunschweig
- α-^{32}P UTP (10 TBq/mmol) — Hartmann analytic GmbH, Braunschweig

2.1.5. Standard oligonucleotides used for cloning

M13 uni (-21) — MWG-Biotech, Ebersberg

5´-TGT AAA ACG ACG GCC AGT-3´

M13 rev (-29) — MWG-Biotech, Ebersberg

5´-CAG GAA ACA GCT ATG ACC-3´

SP 6 — MWG-Biotech, Ebersberg

5´-CA TTT AGG TGA CAC TAT AG-3´

T3 MWG-Biotech, Ebersberg

5´-AAT TAA CCC TCA CTA AAG GG-3´

T7 MWG-Biotech, Ebersberg

5´-TAA TAC GAC TCA CTA TAG GG-3´

T7 term MWG-Biotech, Ebersberg

5´-CTA GTT ATT GCT CAG CGG T-3´

Sequencing primer were labeled with IRD700 or IRD800, respectively.

2.1.6. Antibodies

General primary antibodies used in this work have been described previously (Stöckel and Oelmüller, 2004; Stöckel et al., 2006). Antibodies against AtCnfU-IVb have been described in Yabe et al. (2004). Anti chicken IgG peroxidase conjugated antibodies (Sigma-Aldrich Chemie GmbH, Taufkirchen) served as secondary antibodies.

2.1.7. Bacteria strains and plasmids

E. coli DH5α Invitrogen GmbH, Karlsruhe

E. coli Top10 Invitrogen GmbH, Karlsruhe

E. coli NovaBlue VWR International GmbH, Dresden

E. coli XL1-Blue Stratagene, Amsterdam, Netherlands

The pGEM®-T Vector (Promega GmbH, Mannheim) was used for subcloning.

2.1.8. Plant material

Genetic and biochemical studies were exclusively carried out with *Arabidopsis thaliana* ecotype *Columbia* var. *Col 0*. Ecotypes used for map-based cloning and identified mutants, respectively are indicated in the text.

2.1.9. Standard media

Church buffer: 1 % (w/v) bovine serum albumin, 1 mM EDTA, 0.5 M phosphate buffer, 7 % (w/v) SDS (pH 7.0)

LB medium: 10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl (pH 7.0); for solid medium 1 % (w/v) agar was added

10 x Mops: 200 mM MOPS, 50 mM sodium acetate, 10 mM EDTA (pH 8.0)

PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4 (pH 7.4)

Phenol:Chloroform:Isoamyl alcohol (25:24:1): equilibrated with Tris-HCl (pH 8.0)

20 x SSC: 3 M NaCl, 0.3 M sodium acetate (pH 7.0)

5 x TBE: 54 g/l Tris, 27.5 g/l boric acid, 4.65 g/l EDTA (pH 8.0)

TBS: 140 mM NaCl, 35 mM Tris-HCl (pH 7.4)

10 x TE: 100 mM Tris-HCl, 10 mM EDTA (pH 8.0)

2.2. Methods

2.2.1. Cultivation of organisms

2.2.1.1. Cultivation of bacteria

Chemical competent *E. coli* cells were prepared as described in Hanahan (1983). Bacterial strains were cultivated at 37 ºC either in liquid or solid LB medium containing appropriate antibiotics as selection markers (Sambrook and Russell, 2001). Plasmids were transformed by heatshock treatment (Hanahan 1983).

2.2.1.2. Plant material and growth

Arabidopsis thaliana seedlings were grown under continuous white light of 100 µmol m^{-2} s^{-1} at 22 °C. Seeds were sterilized with 2 % (v/v) sodium hypochlorite and 0.08 % (v/v) N-laurylsarcosinate, washed five times with 1 ml sterile water, and placed on Petri dishes with Murashige and Skoog (1962) media containing 1.35 % (w/v) sucrose. For the first 48 h, the seeds were kept in darkness at 4° C. After 18 d, the seedlings were either harvested or transferred to soil for propagation. Other treatments are indicated in the text. *hcfb12-1* is a chemically induced EMS mutant. *hcfb12-2* is a T-DNA insertion line, N829192, obtained from the Nottingham Arabidopsis Stock Centre, Great Britain (Alonso et al., 2003). Segregation analyses of *hcfb12-2* were performed using the following primer pairs:

5′-AGGGTTTAAAATGAGCTTCAT-3′ and 5′-TCTTTCGTGGATCTACCTGGG-3′.

The T-DNA is inserted into the fifth exon (1304 nt downstream of the ATG codon) of *At3g18680*.

For the illumination experiment, *Arabidopsis* seedlings were grown under white light for 10 days, kept in darkness for 4 days, followed by illumination with white light for 1 day.

2.2.2. Pigment analysis, chlorophyll fluorescence and spectroscopic measurements

2.2.2.1. Chlorophyll measurements

Chlorophyll concentrations were determined as described in Porra et al. (1989).

2.2.2.2. Chlorophyll fluorescence measurements

In vivo chlorophyll *a* measurements were performed with 18 days old *Arabidopsis* seedlings, using a Fluorcam 700 MF (Photon System Instruments, Brno, Czech Republic). The following program was used: 10 min dark-adaptation, 3 s measuring F_o, light pulse of 3000 µmol m^{-2} s^{-1} for 1600 ms to determine F_m, actinic light for 10 min to determine F_t, F_m' and dark relaxation of 2 min for F_o'. The quantum yield of PSII (ΦPSII) was calculated as $\Phi PSII = (F_m'-F_t)/F_m'$. Non-photochemical quenching (NPQ) was calculated as $NPQ = (F_m-F_m')/F_m'$ (Maxwell and Johnson, 2000).

2.2.2.3. 77 K measurements

77 K fluorescence emission spectra were recorded from 600 to 800 nm after excitation at 440 nm, using the Hitachi F-3000 fluorescence spectrophotometer. *Arabidopsis* seedlings were homogenized in 0.33 M sorbitol, 50 mM Hepes-KOH pH 8.0, 1 mM $MgCl_2$, and 2 mM EDTA. Chlorophyll concentrations were adjusted to 1 µg/ml.

2.2.2.4. P_{700} absorbance changes

The light-induced *in vivo* absorbance changes of P_{700} at 810 nm were measured using the PAM101 fluorometer connected to the dual wavelength emitter/detector unit (ED-P700DW, Heinz Walz GmbH, Effeltrich). Saturing far-red light (730 nm, 15 W m^{-2})

emitted by a far-red diode (102-FR, Heinz Walz GmbH, Effeltrich) for 1 min was applied to oxidize P_{700}. After 30 s of far-red light, a strong white light pulse of 6000 µmol m^{-2} s^{-1} was applied for 800 ms. The maximum signal difference (ΔA_{810max}) between the reduced and the oxidized states of P_{700} was used to estimate the photochemical capacity of PSI (Barth and Krause, 2002).

2.2.3. Electron microscopy

Electron micrographs were performed as previously described (Kusnetsov et al., 1998).

2.2.4. RNA analyses

2.2.4.1. RNA isolation

RNA isolation was done as described in Heim et al. (1993). 300 mg plant material was frozen in liquid nitrogen and ground in extraction buffer (1 M Tris-HCl pH 9.0, 10 mM EDTA, 1 % (w/v) SDS, 700 µl phenol). After centrifugation (2 min, 10000 *g*), the aqueous phase was blended with 700 µl phenol. After centrifugation (cf. above), the aqueous phase was precipitated with 2.5 vol. 96 % (v/v) ethanol and 1/10 vol. 3 M sodium acetate. After centrifugation (15 min, 10000 *g*), the pellet was solved in 200 µl water and precipitated with 200 µl 4 M LiCl. After centrifugation (15 min, 10000 *g*), the pellet was washed with 70 % (v/v) ethanol, air-dried and solved in 20 µl water.

2.2.4.2. Northern blot analyses

15 µg total RNA of wild type and mutant plants were loaded per lane. Dilution series are indicated. Gel electrophoresis was carried out using 1.2 % (w/v) agarose gels containing 2 % (v/v) formaldehyde. RNA was transfered to nylon membranes (Hybond-$N^{+™}$ membrane, Amersham Biosciences Europe GmbH, Freiburg) by capillary blotting, and cross-linked under UV irradiation (254 nm, 3 min).

The following primer pairs were used for the amplification of the probes:

psaA-f, 5′-AGGCTTCCACAGTTTTGGTTT-3′	*psaA*-r, 5′-CCCAAACATCTGACTGCATTT-3′
psaB-f, 5′-ACCCCGACTCGAGTAGTCATT-3′	*psaB*-r, 5′-CGCAGCTTGAGTCGTAAAATC-3′
psaC-f, 5′-GAGCATGCCCTACAGACGTA-3′	*psaC*-r, 5′-TTCGAGTTGTTTCATGCCATA-3′
PsaD-f, 5′-ATGGCAACTCAAGCCG-3′	*PsaD*-r, 5′-CTCTTCCTGGATTCGCTTTC-3′
PetC-f, 5′-GATGGCGATGTCAAGTGG-3′	*PetC*-r, 5′-GCTTCATCTATATCCGCGTG-3′
psbA-f, 5′-CTTCTGCAGCTATTGGATTGC-3′	*psbA*-r, 5′-CATTTTCTGTGGTTTCCCTGA-3′
PsbO-f, 5′-CTGCTTCGAGCCTACTTCCTT-3′	*PsbO*-r, 5′-GCAGTGTTCTTCACGTTCTCC-3′
rbcL-f, 5′-GCGTATGTAGCTTATCCC-3′	*rbcL*-r, 5′-TCCCCCTGTTAAGTAGTC-3′
RbcS-f, 5′-CACTATGGTCGCTCCTTTCAA-3′	*RbcS*-r, 5′-GTGAAGCTTGGTGGCTTGTAG-3′
18S rRNA-f, 5′-ACTTATGGAAGGGACGCATTT-3′	*18S rRNA*-r, 5′-CATCCCAAGGTTCAACTACGA-3′

rps14-f, 5′-TCATTTGATTCGTCGATCCTC-3′	*rps14*-r, 5′-ACGTCGATGAAGACGTGTAGG-3′
PsaF-f, 5′-GCAAGGACTCAAAACAGTTCG-3′	*PsaF*-r, 5′-AACTCTCTGTAGGCAGCAACG-3′
PsaH-f, 5′-ATGGCGTCTCTTGCAACC-3′	*PsaH*-r, 5′-TCCTGAGGACCTCTCTTGATG-3′
PsaL-f, 5′-CGAGCTGTTAAATCCGACAAG-3′	*PsaL*-r, 5′-GTGAACTTAGCCCATCCATCA-3′
Hcfb12-f, 5′-TTACCTCTTGCTCCCCAATCT-3′	*Hcfb12*-r, 5′-TCTCTCCTTTGATTGCTTTGG-3′
Hcf101-f, 5′-TGGTATGGGTGCTAGAGTTGG-3′	*Hcf101*-r, 5′-TGGTCGTATGTCCTCAGGTTC-3′
Pyg7-f, 5′-TCGAATCGAACATGGTTCTTC-3′	*Pyg7*-r, 5′-TTATAAAGCTTCACGCGATCC-3′
ycf3-f, 5′-AGGAAATTATGCGGAAGCATT-3′	*ycf3*-r, 5′-TGGTAAAAAGGGGTTTCGTTC-3′

Hybridization probes were labelled with ^{32}P dCTP using the Random Prime DNA labeling kit from Amersham Biosciences Europe GmbH (Freiburg). Church buffer (Church and Gilbert, 1984) was used for hybridization (18 hours, 65 ºC). Membranes were washed for 60 min at 65 ºC using the following buffer: 40 mM sodium phosphat pH 7.0, 2 mM EDTA, 1 % (w/v) SDS. Quantification was done with a phosphorimager (Storm^{TM}, Molecular Dynamics, Freiburg).

2.2.4.3. RT-PCR

cDNA was generated using the reverse transcription polymerase chain reaction (RT-PCR), according to the manufacturer's protocol (RevertAid^{TM} first strand cDNA Synthesis kit, MBI Fermentas GmbH, St. Leon-Rot).

2.2.4.4. Run-On transcription assay

Run-on transcription assays were performed using approximately 2 x 10^7 chloroplasts in 100 µl of 50 mM Hepes-KOH, pH 8.0, 10 mM $MgCl_2$, 25 mM potassium acetate, 10 mM DTT, 55 µg heparin, 125 µM CTP, GTP, ATP, 10 µM unlabeled UTP, and 50 µCi α-^{32}P-UTP (110 TBq/mmol, Mullet and Klein 1987). Incorporation of α-^{32}P-UTP into RNA was determined according to Rushlow and Hallick (1982), and radiolabeled transcripts were hybridized to plasmid DNA on nylon membranes (c.f. Northern blot analyses). Hybridization, washing of membranes and quantification was done as described for Northern blot analyses.

2.2.4.5. Actinomycin D treatment

Arabidopsis seedlings were harvested under water and transferred into a solution containing either 200 µg/ml actinomycin D or water as a control. The seedlings were incubated for 10 min, 1 h, 5 h, 7 h, 10 h before total RNA was isolated (cf. above). Probes for hybridization are listed above.

2.2.4.6. Polysome analyses

Polysomes were isolated as described in Barkan (1993). 0.3 g frozen plant material was ground in liquid nitrogen, and 1 ml polysome extraction buffer (0.2 M Tris-HCl pH 9.0, 0.2 M KCl, 35 mM $MgCl_2$, 25 mM EGTA, 0.2 M sucrose, 1 % (v/v) Triton X-100, 2 % (v/v) polyoxyethylene-10-tridecyl ether, 0.5 mg/ml heparin, 100 mM ß-mercaptoethanol, 100 µg/ml chloramphenicol, 25 µg/ml cycloheximide) was added.

After centrifugation (5 min, 10000 *g*), sodium deoxycholate was added to a final concentration of 0.5 % (w/v). After centrifugation (15 min, 10000 *g*), purified polysomes were fractionated in sucrose gradients (15-55 % (w/w) sucrose in 40 mM Tris-HCl pH 9.0, 20 mM KCl, 10 mM $MgCl_2$, 100 µg/ml chloramphenicol, 0.5 mg/ml heparin), centrifuged for 12 h at 40.000 rpm in a SW 40 TI swing-out rotor (Beckman Coulter Inc., Fullerton, USA). Eleven 1 ml fractions were collected and either RNA (Barkan, 1988) or proteins (cf. below) were isolated. Hybridization, washing of membranes and quantification occurred as described for Northern blot analyses.

2.2.4.7. Primer extension analyses

Primer extension was performed as previously described (Fey et al., 2005).

2.2.5. DNA analyses

2.2.5.1. DNA isolation

Genomic DNA from *Arabidopsis* was isolated as described in Dellaporta et al. (1983). Plasmid DNA isolation occurred as described in Birnboim and Doly (1979).

2.2.5.2. PCR

Analyses using the polymerase chain reaction (PCR) were carried out as described in Sambrook and Russell (2001).

2.2.5.3. DNA sequencing

DNA sequencing reactions were carried out using a Li-COR 2400 sequencer (LI-COR Biosciences GmbH, Bad Homburg), according to the manufacturer's protocol (CycleReader™ DNA sequencing kit, MBI Fermentas GmbH, St. Leon-Rot).

2.2.5.4. Positional cloning of *hcfb12-1*

A segregating F_2 progeny was generated by crosses of male pollen donor plants of heterozygous lines of *hcfb12-1* in *Columbia* (Col 0) background with female recipient plants of ecotype *Landsberg* (Ler), followed by selfing of the resulting F_1 plants. To assign the mutant locus to one of the *Arabidopsis* chromosomes, 30 F_2 plants homozygous for the mutant *hcfb12* locus as well as a combination of simple sequence length polymorphism (SSLP): nga248, nga280, nga111, nga168, nga162, nga6, nga8, nga151, nga76 (Bell and Ecker, 1994) and cleaved amplified polymorphic sequence (CAPS) markers: PhyB and AG (c.f. Arabidopsis.org) were used. For high resolution mapping genomic DNA from single leaves of 1172 individual F_2 plants was isolated. For fine mapping procedures, a combination of three known markers (nga162, SSLP marker, cf. above; nt204, SSLP marker, Arabidopsis.org; Arlim15.1, CAPS marker, Arabidopsis.org) and seven newly developed CAPS markers (listed below) were used.

CAPS markers:

MIG5I

5′-CAGACGATGCATCTGACTTGA-3′ 5′-TTGGTCGTGCTGTATGTTTCA-3′ *Sal*I

MYF24I

5′-TTGATGGTGCATAGCAGAAAA-3′ 5′-ATTAACACCAACGCTCAATGC-3′ *Hinf*I

MYF24II

5′-AGCTCCTTGGAATTTGCATTT-3′ 5′-TCAAAGATTCATCATCGGTAGAGA-3′ *Ssp*I

MVE11I

5′-AACATACTGATGCGAAAGACCA-3′ 5′-GGTACACGAAGACGGAAACAA-3′ *Taa*I

MVE11II

5′-ACAATAACCAGAGCGATCACG-3′ 5′-TCAGACTCTTCACCGCAATCT-3′ *Kpn*I

	MVI11I		
5′-GGTATGGCTTTTTCCGTTGTT-3′	5′-CAAGCGTGACAAATCGAAAGT-3′	*Ssp*I	
	MQC12I		
5′-GCAATGAGCACAAAAAGAAGG-3′	5′-CGTCCAACGAAAAAGAAACA-3′	*Eco*RI	

MIG5I, MYF24I, MYF24II, MVE11II, MVI11I and MQC12I were developed based on the Monsanto Ler Polymorphism Collection, (Arabidopsis.org).

2.2.6. Protein analyses

2.2.6.1. Protein extraction and Western transfer

For immunoblot analysis, leaf material was ground in liquid nitrogen and resuspended in homogenisation buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 2 mM EGTA, and 10 mM DTE). After centrifugation (10 min, 17.000 *g*), the soluble proteins were precipitated with trichloroacetic acid (TCA) and resuspended in gel loading buffer (100 mM Na_2CO_3, 50 mM DTE, and 10 % (w/v) sucrose). Insoluble proteins were resuspended in gel loading buffer. When using total protein extract, isolation was performed as described in Ensminger et al. (2004) Protein gel electrophoresis occurred as described in Schägger and Jagow (1987), and proteins were electroblotted onto PVDF membranes (Amersham Biosciences Europe GmbH, Freiburg).

2.2.6.2. Determination of protein concentration

Protein concentrations were determined as described in Bradford (1976).

2.2.6.3. Antisera analyses

Anti-Hcfb12 antibodies were raised against the following peptides: N-CPRLPSFDGTSKPPL-C and N-CSEPGNIAKAIKGER-C, corresponding to the amino

acids 81-94 and 309-322, respectively (Agrisera, Sweden). For immunoblot analyses affinity purified antibodies with a 1:1000 dilution were used.

2.2.6.4. Isolation of chloroplasts, stromal and thylakoid proteins

Plants were homogenized in isolation buffer (0.3 M sorbitol, 5 mM $MgCl_2$, 5 mM EDTA, 5 mM EGTA, 20 mM Hepes-KOH pH 8.0, 10 mM $NaHCO_3$ and 0.3 mM DTT). After centrifugation (Sorvall® SLA 6000 rotor, 5 min, 2000 *g*), the chloroplast-enriched fraction was purified on sucrose gradients (30-55 % (w/w) sucrose). Chloroplasts were washed with 50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 40 mM ß-mercaptoethanol, and treated with breaking buffer (10 mM Hepes-KOH pH 8.0, 5 mM $MgCl_2$). After centrifugation (Sorvall® SS34 rotor, 10 min, 11900 *g*), thylakoid proteins were resuspended in breaking buffer, stromal proteins were precipitated with TCA, washed with acetone and resuspended in breaking buffer.

2.2.6.5. Sucrose gradient centrifugation

Stromal extracts were isolated (cf. above) and sedimented through sucrose gradients (10-30 % (w/w); Schmitz-Linneweber et al., 2006). After fractionation, proteins were isolated (cf. above) and analyzed by Western blotting.

III. Results

3.1. Characterisation of *hcfb12-1*

3.1.1. *hcfb12-1* shows deficiencies in photosynthesis

A recessive mutant with a high chlorophyll fluorescence phenotype (Figure 1; Meurer et al., 1996; Stöckel and Oelmüller, 2004), *hcfb12-1*, was isolated from an EMS mutagenized *Arabidopsis* seed pool. EMS causes the alkylation of guanine bases, which thereby causes base mispairing, resulting in G/C to A/T transitions (cf. Maple and Møller, 2007). When cultivated on soil, mutant seedlings fail to grow autotrophically. Therefore, *hcfb12-1* seedlings were grown on sucrose supplemented MS-media under a light intensity of 100 µmol m^{-2} s^{-1}. Growth of homozygous *hcfb12-1* seedlings is retarded, and the leaves exhibit a pale-yellowish phenotype (Figure 1).

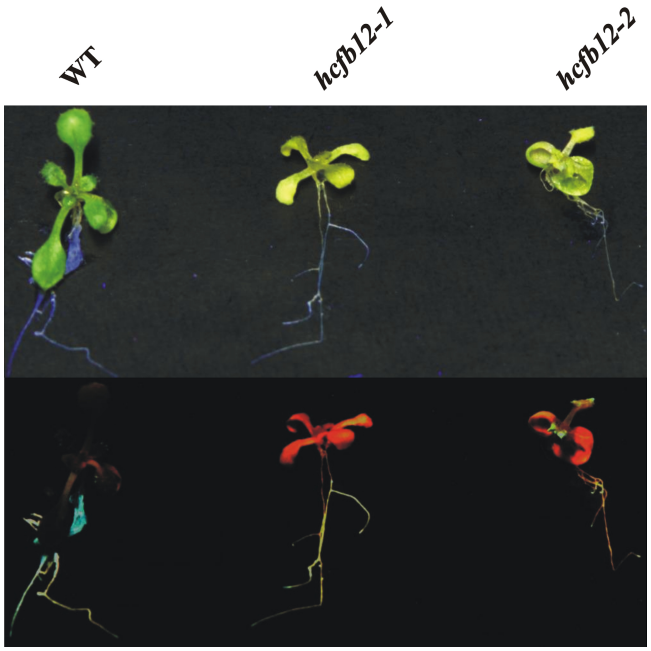

Figure 1: *hcfb12* mutants show deficiencies in photosynthesis. Phenotype of the EMS mutant *hcfb12-1* and the T-DNA insertion line *hcfb12-2* (cf. below) under white light (top) and UV irradiation, respectively (bottom). 18 day old seedlings were grown on Murashige and Skoog (1962) medium containing sucrose under continuous white light of 100 µmol m^{-2} s^{-1}.

Segregation analyses of the *hcfb12-1* alleles in 1172 seedlings revealed a ratio close to the expected 1:3 (26.28 % mutant phenotype to 73.72 % wild type). The result of the chi-square test was calculated as 1.02, confirming the recessive character of the mutation.

3.1.2. Pigment analysis, chlorophyll fluorescence and spectroscopic measurements demonstrate a defect in PSI

Absorbance changes of P_{700} at 810 nm revealed a dramatically reduction of functional PSI in *hcfb12-1* plants (approximately 20 % activity of wild type seedlings; cf. Table 1). 77 K measurements demonstrate a decreased fluorescence and a 2 nm blue shift of the normally observed 735 nm PSI peak (Figure 2), indicating that the energy transfer from PSI antenna to the core is disturbed. The chlorophyll *a*/*b* ratio in mutant seedlings is decreased (Table 1), which is presumably dispositional with the loss of chlorophyll *a* associated proteins. PSII activity was determined by monitoring chlorophyll fluorescence. The F_v/F_m ratio, which reflects the maximum quantum yield of PSII, is decreased in *hcfb12-1*, which is mainly caused by an at least three fold higher F_o value (Table 1). ΦPSII, which reflects the effective quantum yield of PSII is decreased in *hcfb12-1*, although to a lower extent than in other PSI mutants (cf. Table 1; *hcf101*, Stöckel and Oelmüller, 2004). Collectively, these results indicate that the electron transport in *hcfb12-1* is disturbed downstream of PSII, and hence that the mutation might effect PSI.

	Chlorophyll *a*/*b* (mol/mol)	F_v/F_m	Φ PSII	NPQ	ΔA_{810max}
wild type	3.5 ± 0.086	0.869 ± 0.014	0.749 ± 0.027	0.596 ± 0.043	100 % ± 8.696
hcfb12-1	2.8 ± 0.066	0.553 ± 0.051	0.422 ± 0.051	0.485 ± 0.198	19.667 % ± 0.943

Table 1: Pigment analyses and fluorescence measurements of wild type and *hcfb12-1*. F_v/F_m reflects the maximum quantum yield of PSII, ΦPSII the quantum yield of PSII and NPQ the non-photochemical quenching, respectively. ΔA_{810max} reflects the maximum signal difference between the reduced and the oxidized states of P_{700}. Data shown here represent five independent measurements.

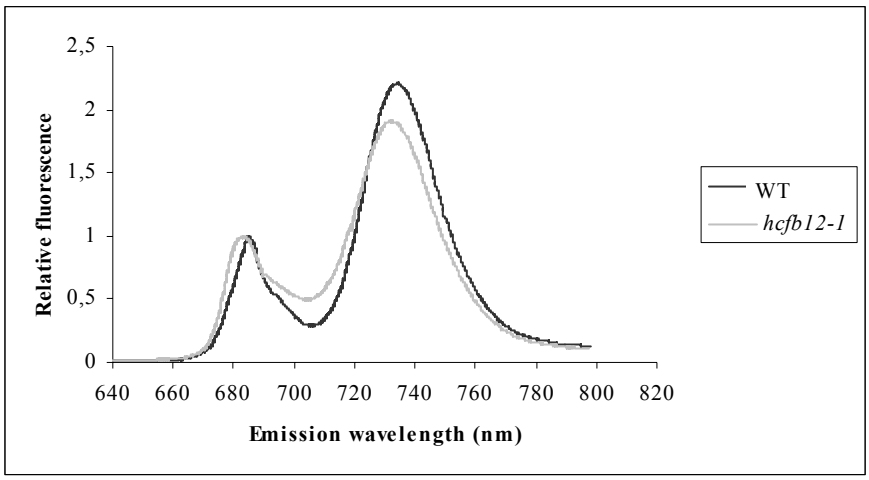

Figure 2: Low temperature fluorescence emission measurements demonstrate a decreased fluorescence and a blue shift of the PSI peak in *hcfb12-1*. 77 K fluorescence emission spectra of wild type (black line) and *hcfb12-1* (grey line) after excitation at 440 nm. Data were normalized to the PSII peak.

3.1.3. Ultrastructure of *hcfb12-1* chloroplasts

Electron microscopy of *hcfb12-1* revealed a significant change in plastid ultrastructure. Stromal lamellae are almost absent in mutant chloroplasts (Figure 3) and grana stacks appear to be swollen. Furthermore the large number of plastoglobuli in the stroma is characteristic for the mutant chloroplasts. Starch granula are not detectable in *hcfb12-1*.

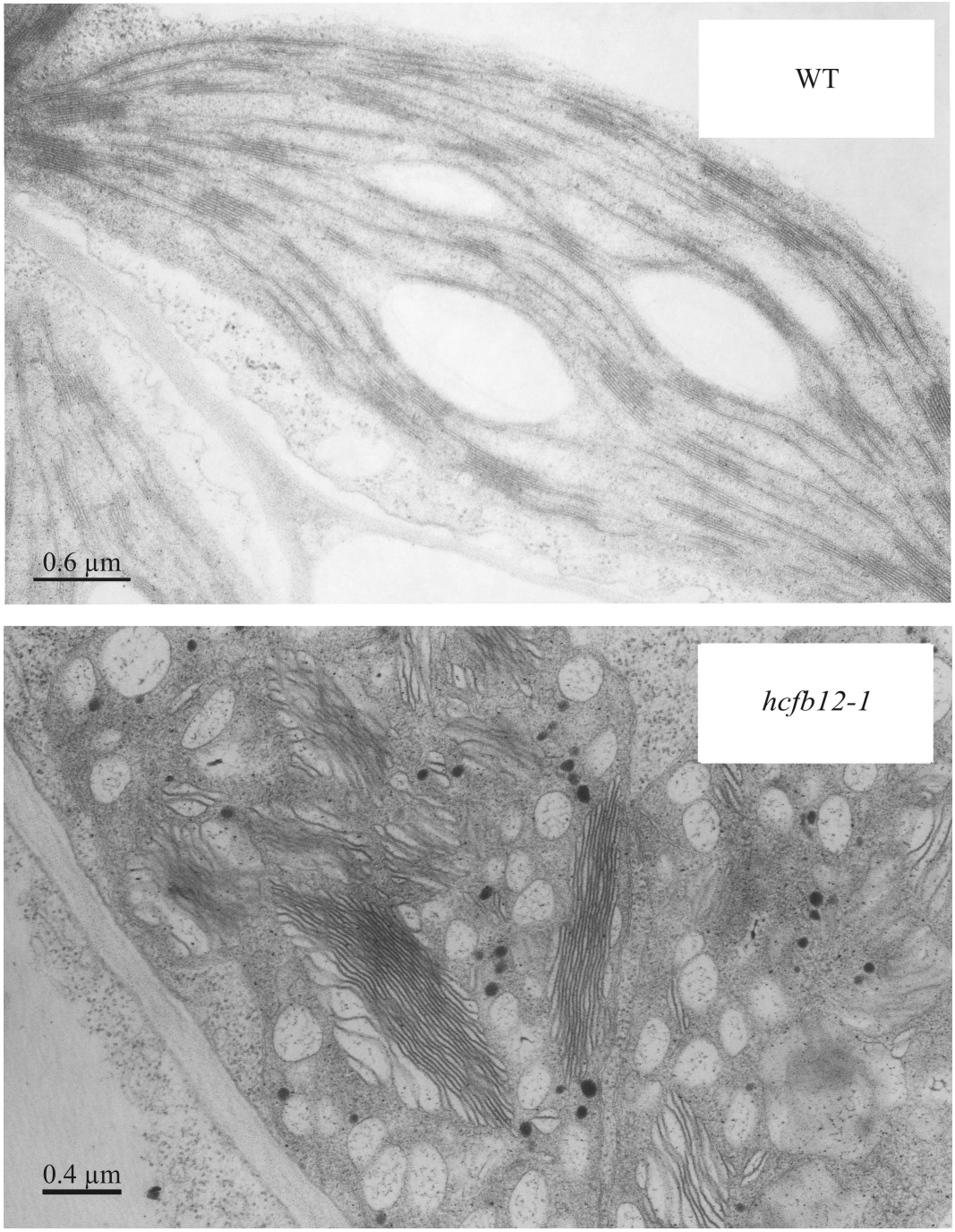

Figure 3: Ultrastructure of wild type and *hcfb12-1* chloroplasts. Bars indicate the scale.

3.1.4. RNA analyses reveal an impairment in the accumulation of transcripts from the *psaA*/*psaB*/*rps14* operon

To assess the transcript levels of photosynthesis genes, RNA gel blot analyses were carried out. Total RNA was isolated from mutant and wild type seedlings and representative genes for either plastid or nuclear encoded subunits of photosynthetic complexes as well as for regulatory proteins were used as hybridization probes. The most remarkable dysfunction of *hcfb12-1* is the failure of proper *psaA*/*B* mRNA accumulation (Figure 4 A). Compared to the wild-type, the 5.2 kb polycistronic *psaA*/*psaB*/*rps14* transcript level is reduced by approximately 75 % in *hcfb12-1*. When a gel was overloaded with *hcfb12-1* RNA to obtain equal *psaA*/*B* signals for the mutant and the wild type, additional messages with sizes higher than 5.2 kb for *hcfb12-1* (Figure 4 C) can be observed. However, primer extension analysis demonstrated that the major transcription start site for the tricistronic *psaA*/*psaB*/*rps14* transcript is identical in *hcfb12-1* and wild-type seedlings and positioned 190 nucleotides upstream of the ATG codon (Figure 4 E). Interestingly, the amount of the mature *rps14* transcript is identical between wild type and *hcfb12-1* (Figure 4 A).

Thus, if the *rps14* transcripts derive exclusively from processing of the tricistronic precursor, this message is either more stable than those of *psaA* and *psaB*, or the lower level of the precursor transcript in *hcfb12-1* is sufficient to generate the same amount of *rps14* transcripts as in wild-type. Alternatively, *rps14* transcripts can also derive also from a *psaA*/*B* independent transcription event.

No alterations in expression levels of the plastid-localized PSI genes *psaC* (Figure 4 A), *psaI* and *psaJ,* as well as nucleus-encoded PSI genes were detectable (Figure 4 A, and data not shown). Transcripts for subunits of the PSII, the Cytb_6/*f*-complex and Rubisco accumulated also to amounts comparable with the wild type (Figure 4 A). Finally, genes for nuclear-encoded regulatory or assembly proteins for PSI, *Hcf101* (Lezhneva et al.,

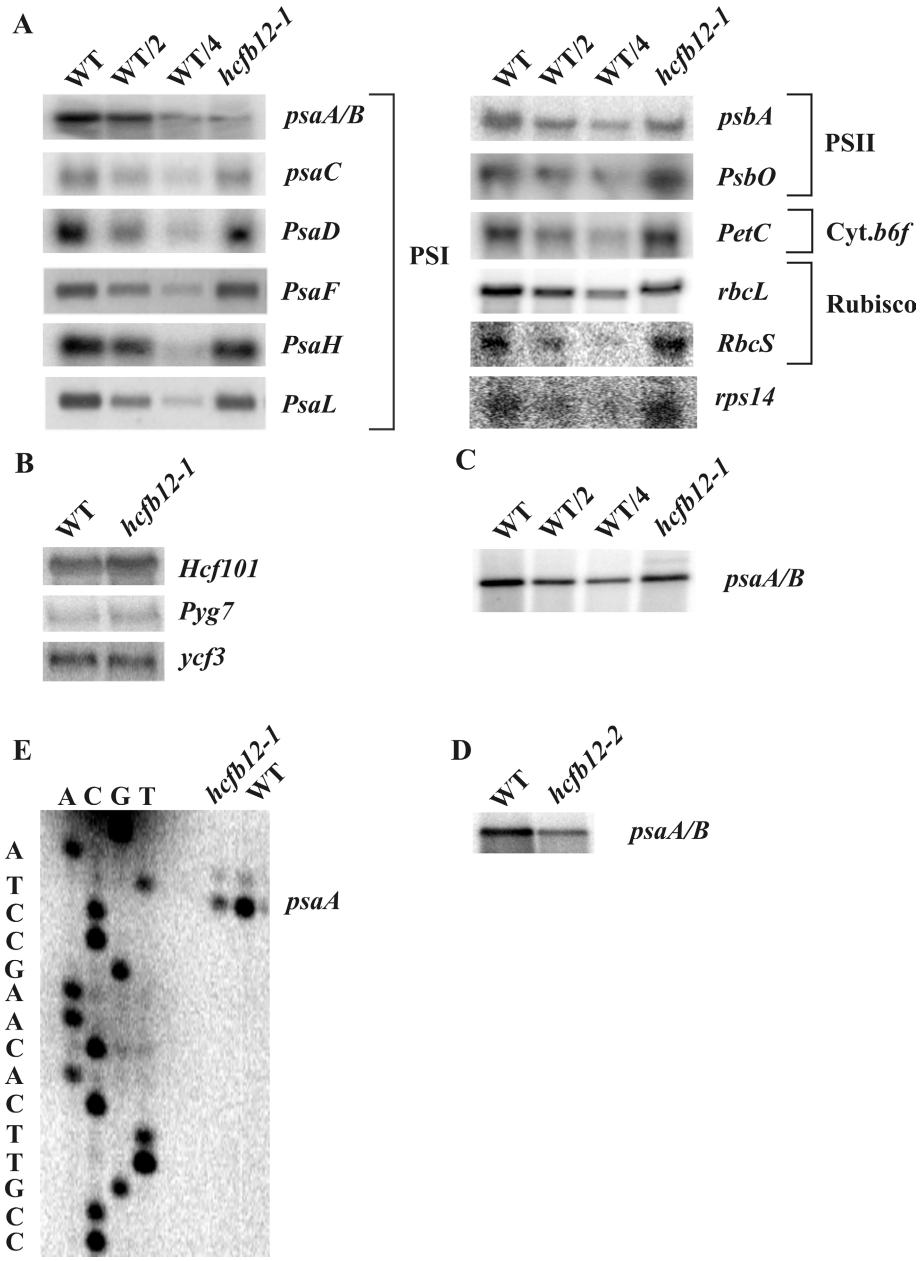

Figure 4: *hcfb12-1* is specifically impaired in the accumulation of *psaA*/*B* transcripts. Northern blot analyses were carried out with total RNA isolated from wild type and mutant seedlings. The following dilutions series were used: WT (15 µg), WT/2 (7.5 µg), WT/4 (3.75 µg); *hcfb12-1* (15 µg). Gene probes for hybridization are indicated. *18S rRNA* was used to confirm equal loading (data not shown). (**A**) Transcript levels of selected photosynthetic components; *rps14* is transcribed as part of the tricistronic *psaA*/*psaB*/*rps14* operon. *hcfb12-1* fails to accumulate proper *psaA*/*B* transcripts. (**B**) Transcripts for regulatory or assembly proteins of PSI. (**C**) Additional messages with sizes higher than 5.2 kb accumulate in *hcfb12-1*. The gel was overload with *hcfb12-1* RNA to obtain equal *psaA*/*B* signals for mutant and wild type. (**D**) *psaA*/*B* transcripts are downregulated in *hcfb12-2*. **(E)** Primer extension analyses of wild type and *hcfb12-1* show the correct *psaA* transcription start at -190 nucleotides relative to the ATG.

2004; Stöckel and Oelmüller, 2004), *Pyg7* (Stöckel et al., 2006) and *ycf3* (Ruf et al., 1997; Naver et al., 2001) were found to be expressed as in the wild type (Figure 4 B). This suggests that *hcfb12-1* is specifically impaired in the accumulation of transcripts from the *psaA*/*psaB*/*rps14* operon.

3.1.5. Protein levels of PSI polypeptide subunits are reduced in *hcfb12-1*

Immunoblot analyses for representative photosynthetic proteins revealed that PsaA is barely detectable in mutant seedlings (Figure 5 A). The extrinsic, integral PSI subunits PsaD, PsaF, PsaL and PsaH were also reduced in *hcfb12-1* (Figure 5 A). This was most dramatic for the ferredoxin interacting subunit PsaD (Figure 5 A) and less apparently for PsaF, PsaH and PsaL (Figure 5 A). In contrast, Lhca1-4 accumulated to levels found in the wild type (Figure 5 A). Moreover, also the amounts of PsbO, Lhcb1 and Lhcb4 from PSII, of PetB and PetD from the Cytb_6/*f*-complex and of AtpB from the ATP synthase (Figure 5 A) remained unaltered in *hcfb12-1*. The absence of PsaA supports the fluorescence data in that PSI is the rate limiting electron transport complex in *hcfb12-1*. Furthermore, the impaired electron transfer is not caused by the reduction of the LhcI/II antennae (Figure 5 A and data not shown).

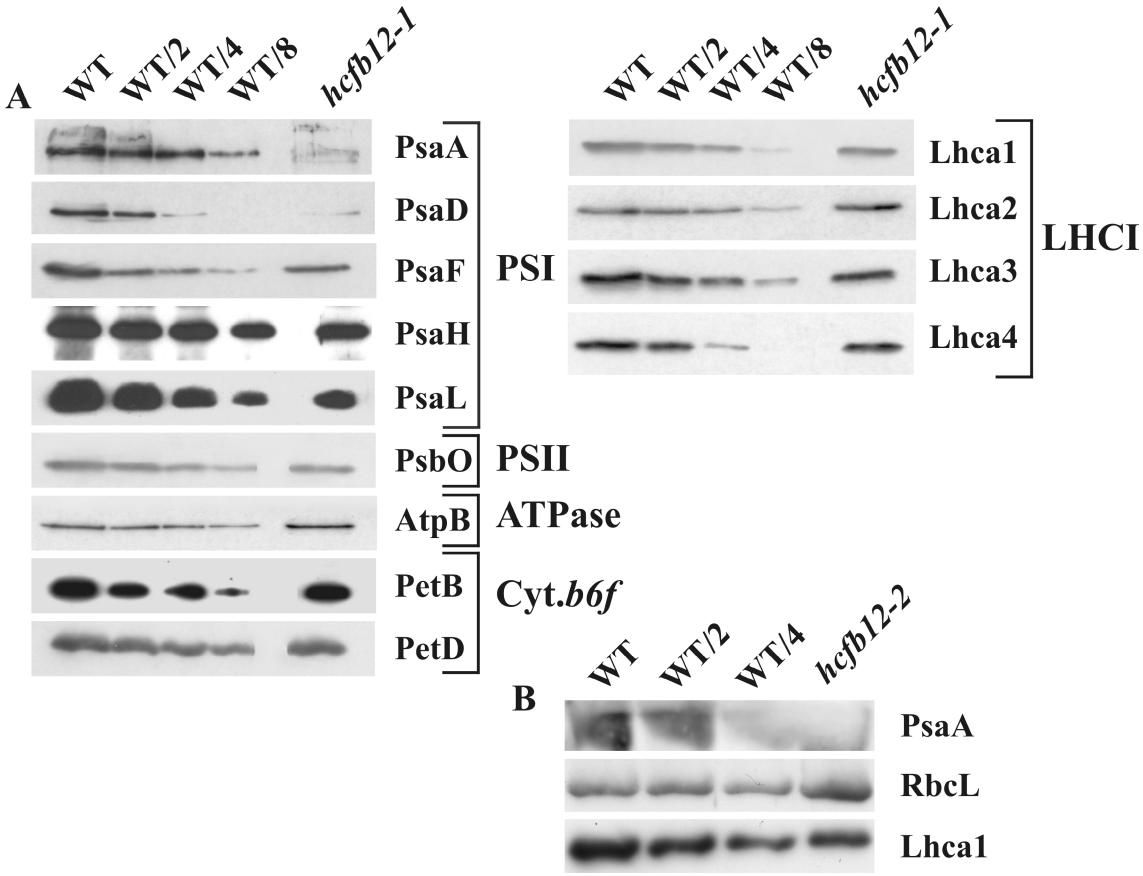

Figure 5: PSI polypeptide levels are downregulated in *hcfb12-1*. (**A**) Western analyses of wild type and *hcfb12-1* seedlings. The following dilutions series were used: WT (15 µg), WT/2 (7.5 µg), WT/4 (3.75 µg), WT/8 (1.86 µg), *hcfb12-1* (15 µg). Antibodies are indicated. PsaA is barely detectable in *hcfb12-1* seedlings. (**B**) Western analyses of wild type and *hcfb12-2* seedlings (dilution series as in (A) without WT/8). Antibodies are indicated.

3.2. Characterisation of *Hcfb12*

3.2.1. Mapping of the EMS mutant *hcfb12-1*

The gene *Hcfb12* has been mapped on chromosome 3, using the molecular markers described in Bell and Ecker (1994) and the markers PhyB and AG (Arabidopsis.org). Next, three known (nga162, nt204 and Arlim15.1) and seven newly developed markers (MIG5I, MYF24I, MYF24II, MVE11I, MVE11II, MVI11I and MQC12I) were used for fine mapping of 1172 individual F_2 plants (cf. Materials and Methods). The markers nga162 and Arlim15.1 enclose the mutant locus with 21 and 32 recombinations, nt204

and MQC12I with 9 and 5 recombinations, MIG5I and MVI11I with two recombinations, and MYF24I and MVE11II with one recombination. The *hcfb12* phenotype was examined by segregation analyses of the following progeny. No recombination event could be observed between the mutation and the marker MYF24II and the marker MVE11I, respectively. MVE11I was found to be located on the same BAC clone and MVE11I is located in a gene next to *hcfb12-1* (*At3g18680*). Sequence analyses of *At3g18680* revealed a single G-to-A conversion at the third exon/intron junction (Figure 6 A). This mutation affected proper splicing of *Hcfb12-1* in the mutant, since a larger transcript for *Hcfb12* in mutant plants in northern analyses can be detected (Figure 6 B). No transcripts with the wild type size were detectable in *hcfb12-1*.

3.2.2. *Hcfb12* mRNA level is light regulated

Hcfb12 was expressed in leaves, but not in roots (Figure 6 B). The *Hcfb12* transcript level was low in etiolated seedlings and upregulated upon illumination (Figure 6 C).

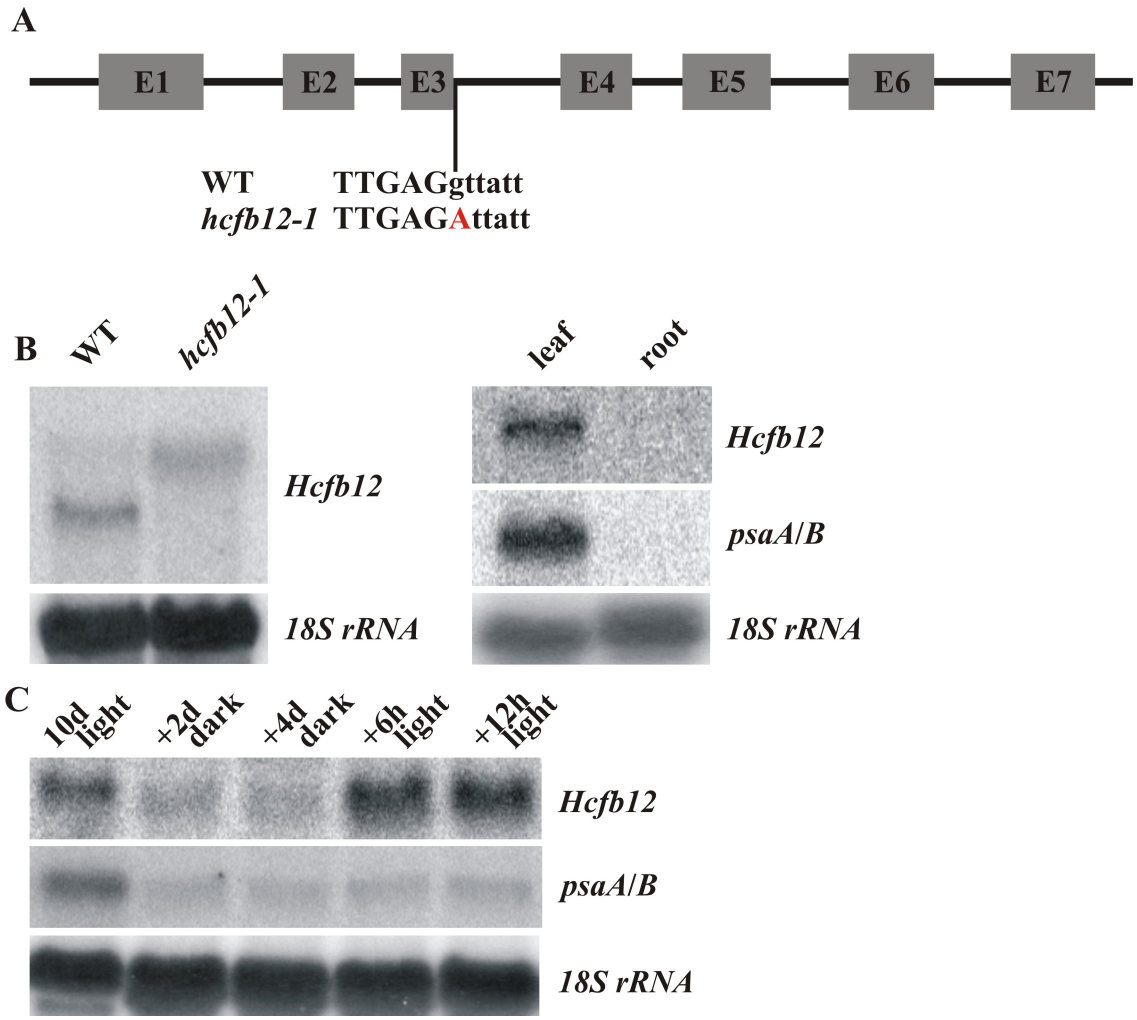

Figure 6: Gene structure of *Hcfb12* (*At3g18680*) and its mRNA expression. (**A**) Genomic structure of *Hcfb12*. G-to-A conversion at the third exon and intron junction in *hcfb12-1* is indicated. Number of exons are indicated. (**B**) Northern blot analyses revealed a splicing defect in *hcfb12-1*, as the result a larger transcript accumulates. Total RNA was isolated and 15 µg were loaded per lane. A *Hcfb12* cDNA probe was used for hybridization. *18S rRNA* confirms equal loading. *Hcfb12* and *psaA*/*B* are not expressed in roots. *18S rRNA* serves as a control. (**C**) Northern blot analyses revealed that *Hcfb12* and *psaA*/*B* transcripts follow a light regulation. *Arabidopsis* wild type seedlings grown under white light for 10 days, were kept in darkness for four days, and then illuminated for 12 hours. Total RNA was isolated and 15 µg were loaded per lane. Hybridization probes are indicated.

3.2.3. *Hcfb12* encodes an evolutionary conserved protein with significant sequence similarities to uridylate kinases

The predicted Hcfb12 polypeptide consists of 340 amino acids with a molecular weight of 36.3 kDa. The N-terminal sequence contains a predicted chloroplast transit sequence of 53 amino acids (reliability class 1, TargetP; Emanuelsson et al., 2000), thus, the mature protein appears to have a molecular weight of approximately 30.5 kDa. A blast search revealed that Hcfb12 exhibits significant sequence similarities to uridylate kinases (UMP kinases) from (plastid-localized) eukaryotic and various prokaryotic organisms. Amongst others, homologous proteins are present in *Oryza sativa* (77 % identities, 86 % positives), *Synechocystis sp. PCC 6803* (65 % identities, 79 % positives), *Escherichia coli* (46 % identities, 67 % positives) and *Bacillus subtilis* (55 % identities, 79 % positives); (cf. Figure 7).

UMP kinases are essential enzymes in the metabolism of pyrimidines. They catalyse the transfer of the γ-phosphoryl group from ATP to UMP. Crystal structure analyses of the *E. coli* UMP kinase revealed several characteristics of the enzyme (Briozzo et al., 2005). Unlike other bacterial nucleoside monophosphate kinases (NMP kinases), UMP kinases exist as hexamers (Briozzo et al., 2005). Moreover, they do not share sequence similarity with NMP kinases and are related to aspartokinases and N-acetylglutamate kinases (Briozzo et al., 2005). The *E. coli* enzyme underlies a complex regulation pattern, such as the activation by GTP and the inhibition by UTP, respectively (Evrin et al., 2007).

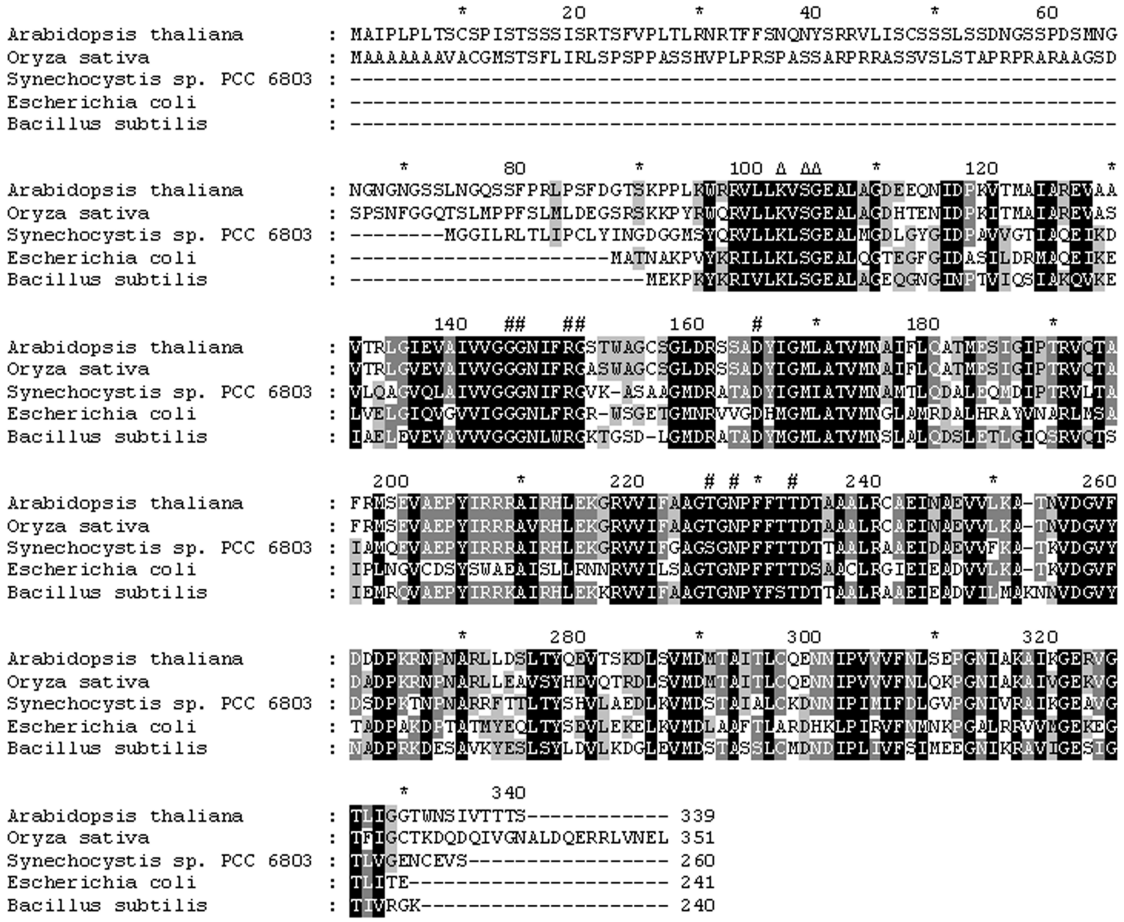

Figure 7: Hcfb12 is an UMP kinase with prokaryotic origin. Sequence alignment of the Hcfb12 protein from *Arabidopsis* (GenBank accession No NP_188498) with related proteins from *Oryza sativa* (accession No. NP_001045493), *Synechocystis sp. PCC 6803* (accession No. NP_441880), *Escherichia coli* (accession No. X78809) and *Bacillus subtilis* (accession No. NP_389533). # indicate residues interacting with UMP and Δ indicate residues interacting with UDP and UTP, respectively (Briozzo et al., 2005). The Hcfb12 polypeptide contains 340 amino acids. The predicted N-terminal chloroplast transit sequence includes the first 53 amino acids for *Arabidopsis* and the first 58 amino acids for *Oryza sativa*, respectively. Thus, the mature *Arabidopsis* protein appears to have a molecular weight of approximately 30.5 kDa.

3.2.4. Analyses of *hcfb12-2* confirm results from *hcfb12-1*

To confirm that the mutation in *hcfb12-1* (*At3g18680*) is responsible for the observed phenotype, the knockout line *hcfb12-2* (N829192) was characterized. PCR analyses of homozygous *hcfb12-2* seedlings confirmed the insertion in the fifth exon of *At3g18680* (data not shown). The insertion line shows the very same phenotype as *hcfb12-1* (Figure

1) and cannot grow without sucrose. *hcfb12-2* analyses confirmed the down-regulation of the *psaA*/*B* transcript level (Figure 4 D) and the absence of the PsaA protein (Figure 5 B).

3.2.5. Hcfb12 is a stroma-localized protein

Polyclonal antibodies were raised against Hcfb12 (cf. Materials and Methods). They detected a polypeptide with a molecular weight of approximately 30 kDa in the stromal fraction (Figure 8 A). No signals were observed in the thylakoid fraction (Figure 8 A). Antibodies against AtCnfU-IVb (Yabe et al., 2004) and PsaF served as stromal or thylakoid controls, respectively (Figure 8 A). No Hcfb12 signal is detectable in *hcfb12-1* (Figure 8 B).

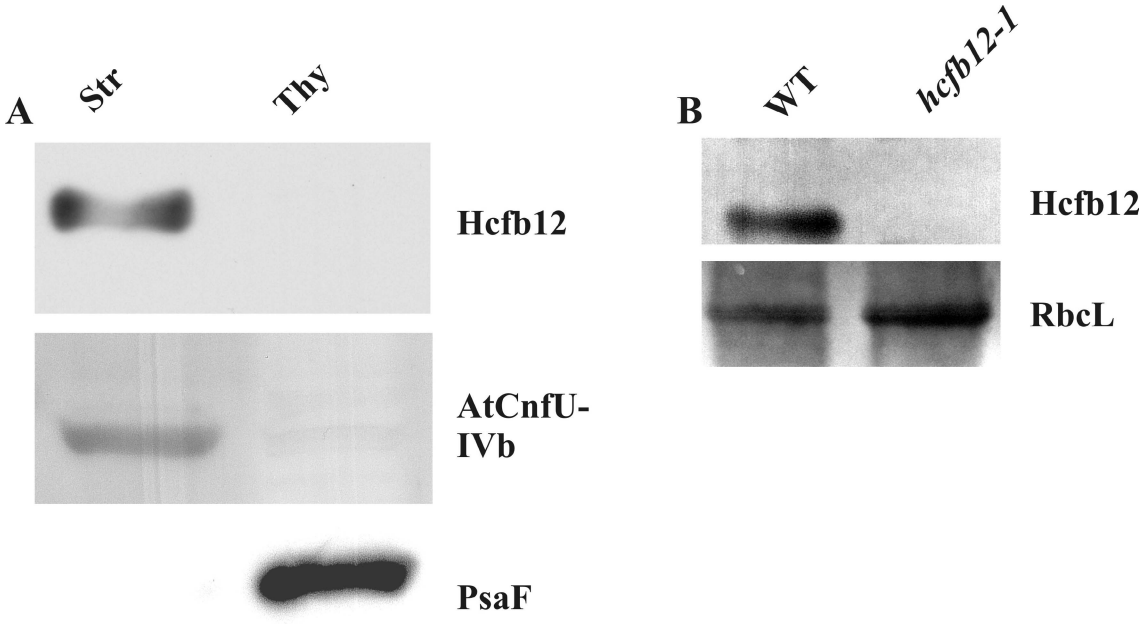

Figure 8: Hcfb12 is localized in the stroma of chloroplasts. (**A**) Stroma and thylakoid fractions were isolated and analyzed by Western blotting with antibodies raised against Hcfb12. 40 µg protein are loaded per lane. Hcfb12 is localized in the stroma of chloroplasts. AtCnfU-IVb (Yabe et al., 2004) serves as stroma control, PsaF as thylakoid control, respectively. (**B**) Western analysis shows absence of Hcfb12 in *hcfb12-1* mutant seedlings. Total protein was isolated prior immunoblotting with antibodies raised against Hcfb12. RbcL serves as loading control.

3.2.6. The *psaA*/*psaB*/*rps14* operon is transcribed *in vitro*

To test whether the reduced *psaA*/*B* mRNA level in *hcfb12-1* is caused by a reduced rate of *psaA*/*B* transcription, *in vitro* run-on transcription assays were performed. Isolated chloroplasts from mutant and wild type seedlings were lysed prior to radiolabeling of the nascent RNA with UTP. Hybridization of the transcripts to selected probes uncovered that the *psaA*/*psaB*/*rps14* operon is transcribed in mutant seedlings. No difference in the intensity of the hybridization signals could be detected to the wild type. The same result was obtained for all other RNAs tested, suggesting that the transcriptional activity of *hcfb12-1* plastids is not affected by the mutation (Figure 9 A). This might suggest that the lower *psaA*/*B* transcript level in *hcfb12-1* is caused by post-transcriptional events.

3.2.7. Transcript stability is unaltered in *hcfb12-1*

psaA/*B* transcript stability in wild type and *hcfb12-1* seedlings was monitored over a period of 10 hours using the transcription inhibitor actinomycin D. In spite of the large difference in the *psaA*/*B* mRNA levels in wild type and mutant seedlings, decay kinetics revealed no differences between 10 min - 10 hours after the application of the substance (Figure 9 B and C). The transcript stability of *psaC*, *psbA*, *trnG* and *16S rRNA* was also not altered in *hcfb12-1* seedlings (data not shown). Thus, it is unlikely that modulation of the stability of transcripts, is a major function of Hcfb12. In this respect, *hcfb12-1* differs from other PSI mutants impaired in *psaA*/*B* mRNA accumulation (Lezhneva and Meurer, 2004).

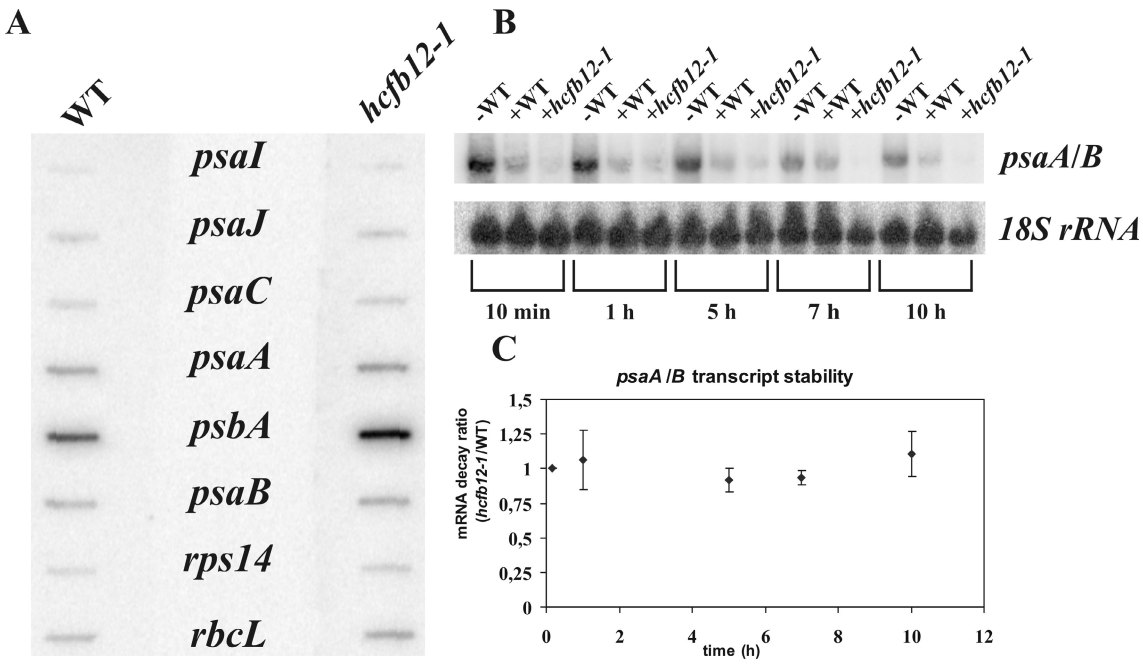

Figure 9: Analyses of transcription rates and transcript stability. (**A**) Run-on transcription assay for wild type and *hcfb12-1* chloroplasts. Approximately 2 x 10^7 chloroplasts were isolated from wild type seedlings and mutant, respectively. Incorporation of α-^{32}P UTP was determined as described in Rushlow and Hallick (1982). Radiolabeled transcripts were isolated (cf. Northern analyses) and hybridized to plasmids, including *rbcL* as control. Quantification occurred with a phosphorimager. (**B**) *psaA*/*B* transcript stability for wild type and *hcfb12-1* mutant seedlings monitored over a period from 10 min to 10 hours using the transcription inhibitor actinomycin D. Seedlings were either treated with actinomycin D (+WT and +*hcfb12-1*) or water (-WT). Seedlings were incubated for 10 min, 1 h, 5 h, 7 h, 10 h prior total RNA isolation and *psaA*/*B* hybridization (cf. Northern analysis). 15 µg RNA were loaded per lane. *18S rRNA* serves as a control. A representative blot is shown as example. (**C**) Statistical analyses of *psaA*/*B* transcript stability. Three independent Northern hybridizations were quantified using a phosphorimager, and the decay ratio for *psaA*/*B* transcripts between wild type and *hcfb12-1* was estimated. A ratio of one indicates equal decay kinetics.

3.2.8. Mutant seedlings are not arrested in translation initiation

To analyse whether Hcfb12 has an effect on polysome loading of the *psaA*/*B* transcripts, ribosomal preparations from mutant and wild type plastids were analyzed by sucrose gradient centrifugations. After fractionation of the gradients, *psaA*/*B* transcripts were detected with northern blot hybridization. As expected, the *psaA*/*B* transcript signals

were significantly weaker in plastid ribosome preparations from *hcfb12-1* (Figure 10). Although the overall distribution of the *psaA*/*B* transcripts in the *hcfb12-1* fractions did not differ dramatically from that of the wild-type, more *psaA*/*B* transcripts from the mutant seedlings are shifted toward the middle and top of the gradient, and thus appear to be less associated with polyribosomes (lane 4-6, Figure 10). Moreover, it appears that mutant seedlings do not compensate the lower *psaA*/*B* transcript abundance by a more efficient polyribosome loading. The distributions of *rbcL* transcripts were found to be equal in *hcfb12-1* and wild type seedlings with a maximum in the fraction 8 (Figure 10).

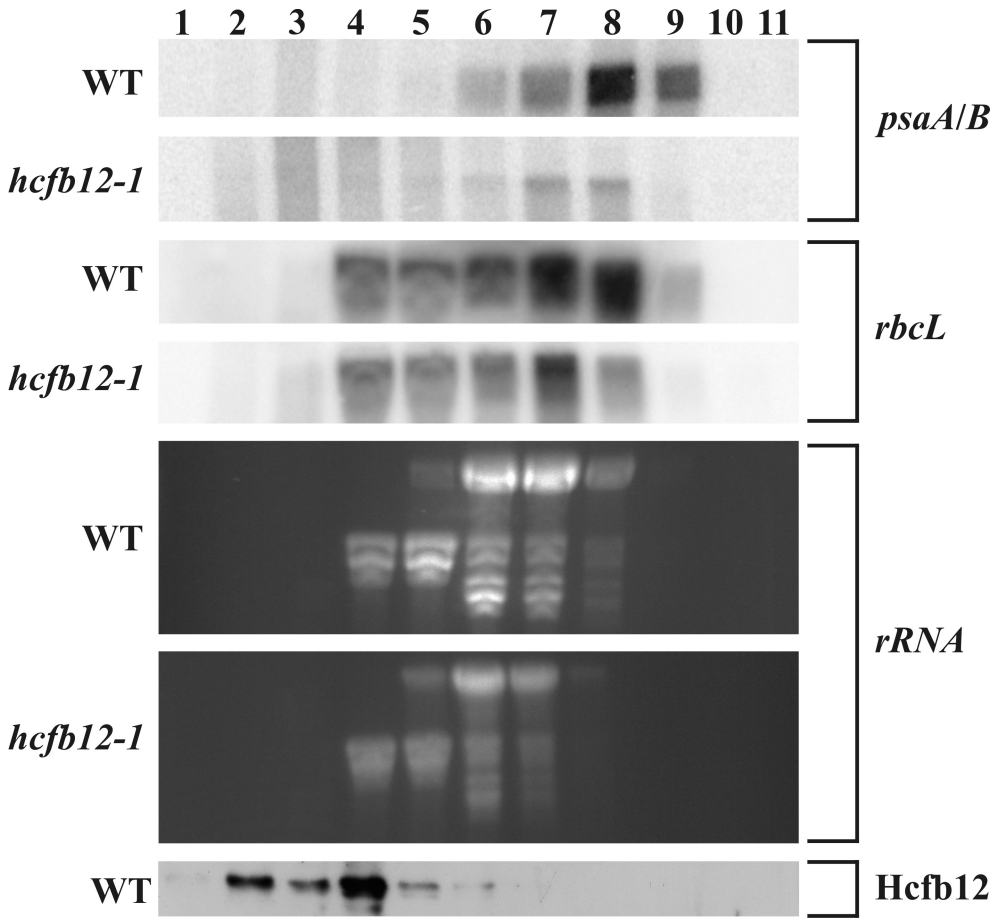

Figure 10: Polysome loading of *psaA*/*B* and *rbcL* transcripts from mutant and *hcfb12-1*. Ribosomal preparations were analyzed in sucrose gradients and fractionated prior total RNA isolation. Transcripts were detected with Northern hybridization. Hybridization probes are indicated. Ribosomal RNA was stained with ethidium bromide. Higher loading of the gradient for *hcfb12-1* was not possible, because it affected the mobility of the polysomes in the gradient. The same fractions were used for protein isolation prior Western blotting with antibodies raised against Hcfb12. Western analyses show that Hcfb12 is not associated with polysomes (bottom).

In addition, it was analyzed whether Hcfb12 is associated with polysomes. Therefore, the ribosomal fractions (cf. above) were analyzed using antibodies against Hcfb12. Signals for Hcfb12 were observed in lanes 1 to 6 with a peak in lane 4. Since no signal can be detected in the polyribosomal fractions (8-10), it appears that Hcfb12 is not stable associated with polysomes (Figure 10).

3.2.9. Sedimentation of Hcfb12 in sucrose gradients

To estimate whether Hcfb12 is associated with high molecular weight complexes, its sedimentation after centrifugation of stromal protein extracts in sucrose gradients was analyzed. The majority of native Hcfb12 is present in fractions with molecular weights lower than Rubisco (approximately 550 kDa, Figure 11).

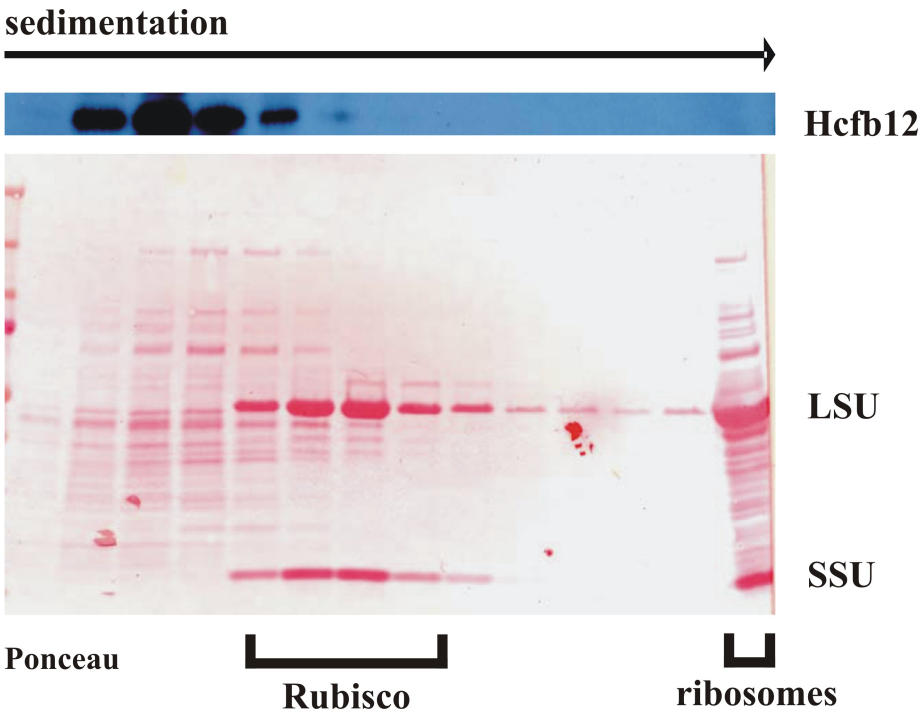

Figure 11: Sedimentation of Hcfb12 in sucrose gradients. Stromal extracts were isolated and analyzed by ultracentrifugation. After fractionation, proteins were isolated and immunoblotted using Hcfb12 antibodies (top). Staining with ponceau shows the sedimentation of Rubisco (LSU and SSU) and the ribosomes (bottom).

However, a small amount of Hcfb12 is also detectable in higher molecular weight complexes within the molecular range of Rubisco. Thus, it is possible that the protein exists as a hexamer (cf. Serina et al., 1995, for the hexameric structure of the homologous protein in *E. coli*), and/or is bound to interaction partner(s).

IV. Discussion

4.1. Functional defects in *hcfb12-1*

Several regulatory proteins required for the biogenesis of PSI in higher plants have been identified in recent years (Ruf et al., 1997; Naver et al., 2001; Yabe et al., 2004; Lezhneva et al., 2004; Stöckel and Oelmüller 2004; Amann et al., 2004; Stöckel et al., 2006; Barneche et al., 2006). Here, the isolation and characterisation of Hcfb12, a novel regulatory protein for PSI, specifically required for *psaA*/*B* transcript accumulation in *Arabidopsis* is described.

P_{700} absorbance changes at 810 nm revealed a dramatic reduction of functional PSI in the mutant seedlings (Table 1). A decreased fluorescence together with a blue shift of the characteristical 735 nm PSI peak at 77 K (Figure 2) was observed (cf. below). Monitoring F_v/F_m and ΦPSII revealed a functional PSII, however, both parameters are decreased in *hcfb12-1*, probably due to secondary effects (e.g. photoinhibition) dispositional with the reduced PSI level (Lee et al., 1996; Haldrup et al., 2003; Stöckel et al., 2006). Western analysis of mutant seedlings demonstrates that the PsaA protein level is at the detection limit (Figure 5 A). In contrast, Lhca1-4, Lhcb1 and Lhcb4 (Figure 5 A and data not shown), selected polypeptide subunits from PSII, the Cytb_6/f- and ATP synthase complex (Figure 5 A) accumulate to levels comparable to the wild type. Collectively, this indicates (i) that the transfer of excitation energy from the LHCI antenna to the PSI core is impaired, (ii) that the impairment of electron transfer is not caused by a reduction of PSII and its LHCII antenna, and (iii) that PsaA, and thus, PSI is the rate limiting electron transport component in *hcfb12-1*. These data are in agreement with the *hcfb12-1* ultrastructure (Figure 3), which shows a reduction of stromal lamellae.

4.2. *hcfb12-1* seedlings fail to accumulate *psaA*/*B* transcripts

Mutants lacking the PSI-A/B dimer generally fail to assemble the entire PSI core complex, although some of the more peripheral subunits can accumulate in the thylakoid membranes (cf. Smart et al., 1991; Cournac et al., 1997; Redding et al., 1999). Known PSI-A/B mutants are either defective in the transcription of the *psaA*/*psaB*/*rps14* operon (Legen et al., 2002) or by the maturation of the transcript (cf. *Chlamydomonas*, Perron et al., 1999; Rivier et al., 2001; Merendino et al., 2006), its stability (Lezhneva and Meurer, 2004) or translation (Amann et al., 2004; Barneche et al., 2006) or the inability to accumulate a stable heterodimer (Lezhneva et al., 2004; Stöckel and Oelmüller 2004; Stöckel et al., 2006). *hcfb12-1* fails to accumulate normal amounts of PSI-A/B because the *psaA*/*B* mRNA level is reduced (cf. Northern analyses and primer extension analysis). Transcription of the tricistronic operon (detected *in vitro*), the stability of the detectable message over a period of approximately 10 h, and its association with polysomes might not be the primary targets of Hcfb12 (Figure 9 and 10). Thus, it is possible that a process between transcription initiation and accumulation of the stable transcript might be affected in *hcfb12-1*. To date, *hcfb12-1* is the first identified mutant in *Arabidopsis* that is affected in such early events of *psaA*/*B* transcript accumulation.

The role of transcriptional regulation in plastid gene expression caused by multiple RNA polymerases, σ factors and other transcription factors (Pfannschmidt and Link 1994; Sugita and Sugiura 1996; Shiina et al., 2005), and many nuclear encoded genes required for plastid gene expression have been identified. For instance, the low *psbA* mRNA level in *ptac2* was shown to be caused by a reduced transcription rate (Pfalz et al., 2006). Run-on transcription assays in *hcfb12-1* showed that incorporation of radiolabeled UTP into transcripts deriving from the polycistronic *psaA*/*psaB*/*rps14* operon was not affected in *hcfb12-1*. Thus, the transcription initiation in *hcfb12-1* plastids is not altered at least in an *in vitro* assay (Figure 9 A). This might suggest that

the lower *psaA*/*B* transcript abundance in *hcfb12-1* is either caused by transcriptional events, which are not detectable *in vitro*, or by early post-transcriptional events.

Various post-transcriptional processes affecting RNA splicing, editing, processing and stability are controlled by nuclear factors and several higher plant mutants have been identified with defects in these processes (Barkan and Goldschmidt-Clermont, 2000). For instance, *psaA*/*B* transcripts (amongst others) are less associated with polyribosomes in the nuclear maize mutant *cps2*, indicating a general defect in translation initiation (Barkan, 1993).

Raa1, Raa2 and Raa3 are required for *psaA trans*-splicing in *Chlamydomonas*, whereas Tab1 and Tab2 are required for translation initiation of *psaB* (reviewed in Rochaix et al., 2004). The *Arabidopsis* mutant *apo1* might be affected in translational elongation of *psaA*/*B* transcripts (Amann et al., 2004). *psaA*/*B* and *psbD*/*C* transcripts are less associated with polysomes in the *atab2-1* mutant (Barneche et al., 2006). In contrast to all these mutants, in which the *psaA*/*B* message is not efficiently translated, the *psaA*/*B* message in *hcfb12-1* is stable over the monitored period of time (Figure 9 B and C). In *hcfb12-1* seedlings the *psaA*/*B* messages are associated with high molecular mass polyribosmes, although to a lesser extent as in the wild-type (Figure 10). This might have different reasons; one of them could be the severely reduced amount of *psaA*/*B* transcripts in *hcfb12-1*. Polyribosome loading of *rbcL* (Figure 10), *atpB* and *psaC* transcripts (data not shown) in *hcfb12-1* is comparable to that in the wild type. Thus, a general defect in translation initiation in *hcfb12-1* can be excluded. It can be concluded that inefficient translation is most probably also not the primary reason for the reduced message level in *hcfb12-1*. Furthermore, Hcfb12 is not found in the polysomal fraction. Factors affecting the translation efficiency of plastid transcripts are often associated with larger complexes (Lennartz et al., 2006; Schult et al., 2007). Hcfb12 might act on *psaA*/*B* transcripts at an earlier time point than translation initiation. This is consistent with the absence of Hcfb12 in polysomal

(Figure 10) and ribosomal fractions (Figure 11), and with a possible bifunctionality of the enzyme (cf. below).

Interestingly, the amount of the mature *rps14* transcript is identical in wild type and mutant seedlings, while the amount of the tricistronic *psaA*/*psaB*/*rps14* precursor transcript is dramatically reduced in the mutant (Figure 4 A). Similar results have been described for other mutants (Lezhneva and Meurer, 2004; Pfalz et al., 2006). Besides the possibility of an independent transcription of *rps14*, this can be caused by differences in the stability or processing of the precursor *versus* the processed transcript, or the amount of the precursor transcript does not limit the generation of the processed *rps14* transcripts in *hcfb12-1*.

4.3. Evolutionary conservation of prokaryotic UMP kinases

Among prokaryotic (photosynthetic and non-photosynthetic) organisms UMP kinases (named PyrH in prokaryotic systems) are remarkably conserved (Figure 7). Prokaryotic UMP kinases have been of interest because UMP kinase mutants are arrested in cell proliferation in both gram-negative (*Escherichia coli*, Yamanaka et al., 1992) and gram-positive bacteria (*Streptococcus pneumoniae*, Fassy et al., 2004) and are believed to lack an eukaryotic counterpart. Thus, prokaryotic UMP kinases have been discussed as antibacterial targets. Recently, the crystal structure of the *Escherichia coli* UMP kinase was solved, and its hexameric structure is unique among prokaryotic UMP kinases (Briozzo et al., 2005). Bacterial UMP kinases are atypical in both missing sequence similarity and structural relation to other NMP kinases (Briozzo et al., 2005). Unlike prokaryotic UMP kinases, eukaryotic UMP kinases contain a glycine-rich region (GGPG(S/A)GK) at the N-terminus (Zhou and Thornburg, 1998), a sequence which is also present in cytosolic UMP kinases from *Arabidopsis* (e.g. in *At5g26667*, an UMP kinase that lacks a predicted chloroplast transit sequence) but which is not present in Hcfb12. Figure 7 shows substantial

sequence similarities between chloroplast localized UMP kinases from *Arabidopsis* and *Oryza sativa* and UMP kinases from *Escherichia coli*, *Bacillus subtilis* and *Synechocystis*. Moreover, database analyses revealed no other UMP kinases in *Arabidopsis* with a predicted chloroplast transit sequence except Hcfb12 (data not shown). Thus, these data strongly suggest that Hcfb12 is the only (plastid-localized) UMP kinase with prokaryotic origin in *Arabidopsis*.

4.4. Hcfb12, a protein with moonlighting features?

Analyses of UMP kinase mutants in *Escherichia coli* suggest that the enzyme has an additional function beside its catalytic UMP kinase property. The *Escherichia coli* UMP kinase was found to participate in the regulation of the *carAB* operon (Kholti et al., 1998). The enzyme acts as a sensor of the intracellular pyrimidine pool and couples *carAB* gene expression to the availability of pyrimidines in the cell (Kholti et al., 1998). Similarly, the *pyr* operon in *Bacillus subtilis* is under control of the cellular pool of pyrimidines, *via* UMP dependent binding of PyrR to the anti-antiterminator (Lu et al., 1996). Also, a nucleoside diphosphate kinase functions as human *c-myc* transcription factor (Postel et al., 1993). Finally, the aconitase from *Bacillus subtilis* was shown to be a bifunctional protein with aconitase activity when iron is available, and RNA binding activity under iron starvation conditions (Alen and Sonenshein, 1999). Examples for proteins with two unrelated functions are also involved in *psaA* mRNA metabolism. Maa2, a factor involved in *trans*-splicing of the second *psaA* intron in *Chlamydomonas*, shares sequence similarity to pseudouridine synthases, and exchange of several amino acids residues that are essential for pseudouridine synthase activity did not alter *trans*-splicing (Perron et al., 1999). Similarly, Crs2, a factor required for group II intron splicing in maize chloroplast, is related to peptidyl-tRNA hydrolase enzymes, and complementation experiments with *Escherichia coli* suggest a lack of peptidyl-tRNA hydrolase activity (Jenkins and Barkan, 2001). It remains to be determined whether Hcfb12 has an additional function in *psaA*/*B* transcript

processing besides its role in phosphorylation of UMP, and therefore can be added to the list of moonlighting proteins (reviewed by Moore 2004; Figure 12). Because of the presence of UMP kinases in both cell compartments cytosol and chloroplasts, *in vivo* experiments were no suitable tool for determination of the Hcfb12 kinase activity, since the measured kinases activity in chloroplast preparations might particular also derive from cytosolic contaminations. Thus, a recombinant approach was chosen for determination of the kinase activity. However, no reasonable results were achieved by overexpressing Hcfb12 in a prokaryotic system. At the moment, experiments are in progress to overexpress the protein in an eukaryotic system (yeast) followed by biochemical determination of the UMP kinase activity of Hcfb12.

In general, moonlighting proteins exhibit a catalytic function, and in addition, they feature a non-enzymatic, structural or regulatory function (Copley 2003), which often involves the interaction with nucleic acids (Moore 2004). The existence of these enzymes has been interpreted as development towards complexity without expansion of the genome (Copley 2003). This is well demonstrated for plant enzymes involved in the nucleotide metabolism. In particular, this was shown for *Arabidopsis* nucleoside diphosphate kinases (NDPKs), enzymes that catalyze the transfer of the γ-phosphoryl group from ATP to nucleoside diphosphates. The *Arabidopsis* NDPK2 for instance contributes to light signalling processes (Choi et al., 1999), and interestingly, the gene expression of NDPK3 was found to be dependent on photosynthetic redox signals as revealed by array analyses (Fey et al., 2005).

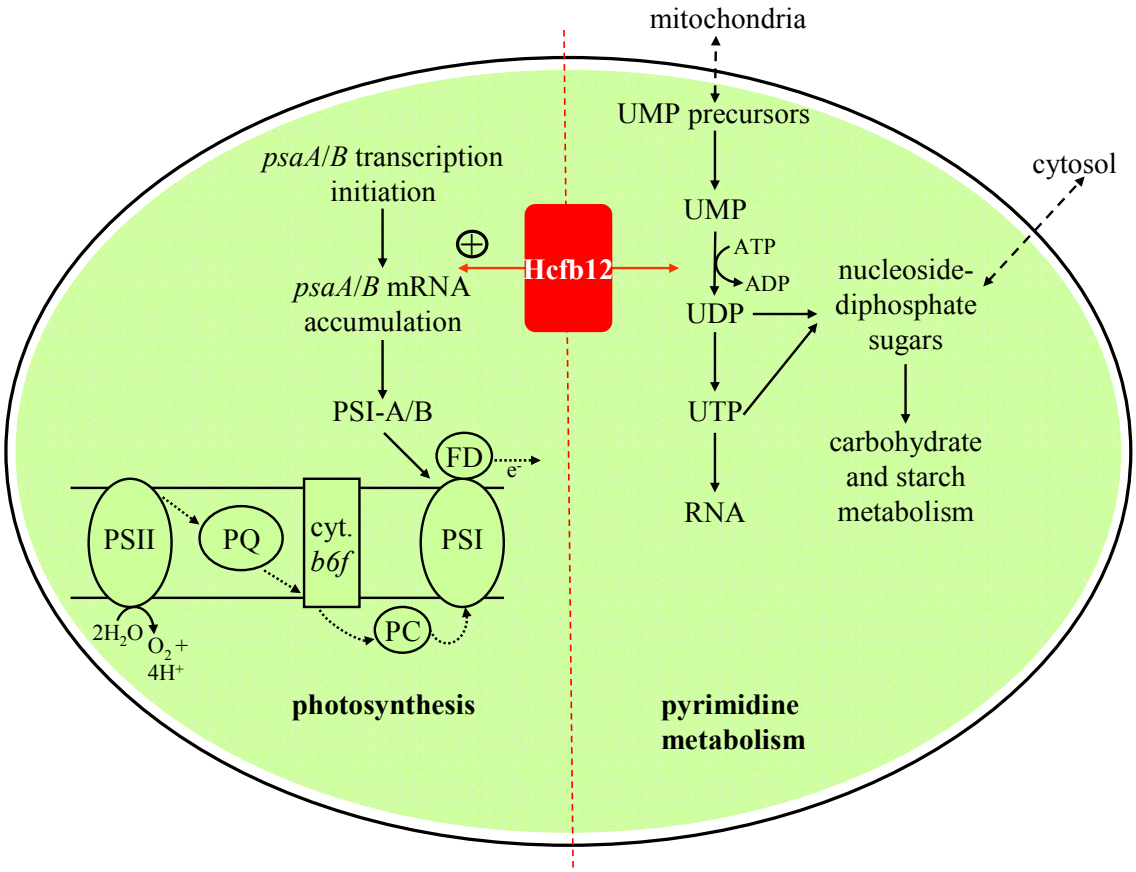

Figure 12: A model representing the possible moonlighting nature of Hcfb12 in the stroma of chloroplasts. *Hcfb12* encodes a evolutionary conserved protein with significant sequence similarities to prokaryotic UMP kinases. UMP kinases catalyse the transfer of the γ-phosphoryl group from ATP to UMP. UMP serves as precursor for the pyrimidine metabolism, and among this, UMP links the pyrimidine metabolism with the carbohydrate metabolism since a large fraction of uridine nucleotides is bound as nucleoside diphosphate sugars (UDP-glucose). Beside its catalytic function, Hcfb12 might exhibit an additional regulatory function. It might act on *psaA/B* transcript accumulation shortly after transcription initiation. Enzymatic reactions of the UMP precursors require the interplay between mitochondria and chloroplast (Zrenner et al., 2006). The major pool of nucleoside-diphosphate sugars (UDP-glucose) is located in the cytosol (Zrenner et al., 2006).

Phosphorylation of UMP is of fundamental importance for plastids, since UMP serves as the precursor for the entire pyrimidine metabolism, and a large fraction of uridine nucleotides is bound as nucleoside diphosphate sugars (UDP-glucose). Thus, the PSI activity might (also) be linked to the carbohydrate metabolism *via* this enzyme. Considering that major UDP and UTP pools are found in the cytoplasm (cf. Zrenner et al., 2006) and that (uridine) nucleotides can be exchanged between

cytosol, mitochondria and the plastid stroma (Zrenner et al., 2006), only a plastid-localized enzyme involved in uridine nucleotide metabolism can couple the carbohydrate metabolism to the photosynthetic electron transport. A shortage of pyrimidines in the plastids can be compensated by importing the nucleotides from the cytoplasm to ensure normal transcription. The presence of a plastid-localized UMP kinase besides the enzymes located in other cellular compartments clearly indicates that the organellar UMP metabolism is important for the plant. It can be proposed that this can be explained by a link of a plastid-localized enzyme to the efficiency of the photosynthetic electron transport. Since limitations in the electron transport may affect many metabolic processes in the plastids, including starch synthesis, which is coupled to the availability of UMP/UDP/UTP, it is also possible that limitations in any of these metabolites (or accumulation of UMP) may downregulate photosynthesis primary by reducing the amount of PSI, which delivers electrons to compounds of the dark reaction.

V. Summary

PSI is a pigment-protein complex located in the thylakoid membranes of cyanobacteria and chloroplasts of algae and higher plants, which functions as a plastocyanin (or cytochrome c_6)-ferredoxin oxidoreductase. In higher plants, the complex consists of 15 different protein subunits; ten of them are encoded in the nucleus (PSI-D/E/F/G/H/K/L/N/O/P) and the residual five (PSI-A/B/C/I/J) are plastid-encoded. The initial step in PSI biogenesis is the formation of the heterodimer PSI-A/B. In order to elucidate novel regulatory mechanism during early events in PSI biogenesis, an EMS mutagenized *Arabidopsis* seed pool was screened for mutants defective in *psaA*/*B* transcript accumulation, and the recessive mutant *hcfb12-1* that fails to grow autotrophically, was isolated.

The *hcfb12-1* mutant shows reduced P_{700} absorbance changes at 810 nm, and a decreased 77 K fluorescence accompanied by a blue shift of the 735 nm PSI peak compared to wild type. In contrast to this, PSII was found to be functional. Therefore, PSI is the rate limiting electron transport component in *hcfb12-1*.

Stroma lamellae are almost absent in *hcfb12-1* chloroplasts, starch granula are not detectable. Thus, ultrastructure analysis corroborated the findings derived by spectroscopic measurements.

hcfb12-1 is specifically impaired in the accumulation of transcripts from the *psaA*/*psaB*/*rps14* operon. The amount of the mature *rps14* transcript is similar in wild type and mutant, indicating an independent regulation of the mature transcript. Moreover, no alterations in expression levels of the remaining PSI genes, transcripts for subunits of PSII, the Cyt*b*$_6$/*f*-complex and Rubisco exist.

In western analyses of *hcfb12-1*, the PsaA protein is at the detection limit. The protein levels of the extrinsic and integral PSI subunits are reduced, whereas no alterations in

protein levels were found for Lhca/b proteins and subunits of PSII, the Cytb_6/*f*-complex and the ATP synthase, respectively. Thus, the genetic lesion is specifically affecting PSI accumulation.

The gene *Hcfb12* (*At3g18680*) has been mapped on chromosome 3. The gene structure consists of seven exons. A single G-to-A conversion at the third exon/intron junction affects splicing of *Hcfb12-1* in the mutant, resulting in the accumulation of a larger transcript giving rise to a non-functional protein product. The *Hcfb12* mRNA level in wild type is light regulated, and transcripts are only detectable in leaves.

Hcfb12 encodes an evolutionary conserved protein with high sequence similarities to prokaryotic uridylate (UMP) kinases and possesses a N-terminal chloroplast transit sequence (reliability class 1, TargetP). Antibodies were raised against Hcfb12 and the protein was immunologically detected in the stroma of the chloroplast with a molecular weight of approximately 30 kDa. No signal is detectable in the *hcfb12-1* mutant.

Northern and Western analyses of an independent T-DNA knockout line, *hcfb12-2* (obtained from NASC), confirmed the results from *hcfb12-1*. The insertion line shows the same phenotype as *hcfb12-1*.

The initiation of *psaA*/*B* transcription in *hcfb12-1* is not impaired. Thus, the lower *psaA*/*B* transcript abundance in *hcfb12-1* might either be caused by the transcriptional process, which is not detectable *in vitro*, or by post-transcriptional events.

The *psaA*/*B* transcript stability remained unaltered in *hcfb12-1*. This might suggest that the mutation affects *psaA*/*B* transcripts at a step between transcription initiation and stable accumulation of the messages.

Mutant seedlings are not arrested in translation initiation, since *psaA*/*B* messages are associated with polyribosomes. The Hcfb12 protein is, however, not detectable in (poly)ribosomal fractions.

Two distinct possible roles of Hcfb12 in the stroma of the chloroplast (i) catalysing the transfer of the γ-phosphoryl group from ATP to UMP, and (ii) regulating *psaA*/*B* transcript accumulation after transcription initiation were integrated in a model.

VI. Thesen

1. Photosystem I ist die limitierende Elektronentransport-Komponente in der Ethylmethansulfonat-Mutante *hcfb12-1* (*high chlorophyll fluorescence*). Sie zeigt eine verminderte P_{700}-Absorption bei 810 nm und eine verringerte 77 K-Fluoreszenz, begleitet von einer Blauverschiebung des PSI-Maximums im Vergleich zum Wildtyp. Die Mutante besitzt jedoch ein funktionsfähiges Photosystem II.
2. Die Anzahl der Stromalamellen in den Chloroplasten der Mutante *hcfb12-1* ist reduziert; Stärkegranula treten nicht auf.
3. Die Mutante *hcfb12-1* ist spezifisch beeinträchtigt in der *psaA*/*psaB*/*rps14*-Transkriptakkumulation. Die Menge des prozessierten *rps14*-Transkriptes ist dagegen zwischen Mutante und Wildtyp gleich. Dies lässt eine unabhängige Regulation des prozessierten *rps14*-Transkriptes vermuten. Alle weiteren untersuchten Gene, die für PSI-, PSII-, Cytb_6/*f*- und Rubisco-Untereinheiten kodieren, zeigen keine Unterschiede im mRNA-Expressionsspiegel zwischen Mutante und Wildtyp.
4. Die Menge an PsaA-Protein in der Mutante *hcfb12-1* ist signifikant reduziert. Auch die relativen Proteinmengen der extrinsischen und integralen PSI-Untereinheiten sind in der Mutante verringert. Dagegen sind die Proteinmengen der Lhca/b-Proteine, der PSII-, Cytb_6/*f*- und ATP-Synthase-Untereinheiten nicht reduziert. Somit ist spezifisch die Photosystem I-Akkumulation durch die genetische Läsion beeinträchtigt.
5. Das Gen *Hcfb12* (*At3g18680*) wurde auf Chromosom 3 kartiert. Der Spleißvorgang von *Hcfb12-1* wird in der Mutante durch eine G-zu-A-Transition innerhalb des Übergangs vom dritten Exon zum Intron verhindert, wodurch ein größeres Transkript akkumuliert. Der *Hcfb12*-mRNA-Spiegel im Wildtyp

unterliegt einer lichtabhängigen Regulation. *Hcfb12* Transkripte sind nur in Blättern nachweisbar.

6. *Hcfb12* kodiert ein evolutionär konserviertes Protein, welches hohe Sequenzähnlichkeit zu prokaryotischen UMP-Kinasen aufweist und eine N-terminale Transitsequenz zum Import in den Chloroplasten besitzt. Gegen Hcfb12 wurden Antikörper erstellt. Das Protein ist immunologisch im Stroma von Chloroplasten mit einem Molekulargewicht von 30 kDa nachzuweisen. Das Protein ist in der Mutante *hcfb12-1* nicht zu detektieren.
7. Northern- und Westernanalysen der unabhängigen T-DNA *knockout*-Linie, *hcfb12-2*, bestätigen die Ergebnisse der Analysen von *hcfb12-1*. Die T-DNA Linie zeigt einen identischen Phänotyp.
8. In der Mutante *hcfb12-1* ist die Initiation der *psaA*/*B*-Transkription nicht beeinträchtigt. Die geringere *psaA*/*B*-Transkriptabundanz wird entweder durch transkriptionelle Prozesse verursacht, welche *in vitro* nicht zu detektieren sind, oder durch posttranskriptionelle Prozesse hervorgerufen.
9. Die Stabilität der *psaA*/*B*-Transkripte in der Mutante *hcfb12-1* ist vergleichbar zu der des Wildtypes. Eine Beeinflussung der *psaA*/*B*-Transkripte durch die Mutation betrifft vorwiegend den Schritt zwischen der Transkriptionsinitiation und mRNA-Akkumulation.
10. *psaA*/*B*-Transkripte sind in der Mutante *hcfb12-1* mit Polyribosomen assoziiert. Somit ist die Mutante zur Translationsinitiation von *psaA*/*B*-Transkripten befähigt. Das Protein Hcfb12 ist in (poly)ribosomalen Fraktionen nicht nachweisbar.
11. Zwei mögliche Funktionen von Hcfb12 im Stroma des Chloroplasten (i) Katalyse des Transfers der γ-phosphoryl-Gruppe von ATP auf UMP und (ii) die Regulation der *psaA*/*B*-Transkriptakkumulation nach der Transkriptionsinitiation wurden in ein Modell integriert.

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Publications

Stöckel J, Bennewitz S, Hein P, Oelmüller R (2006) The evolutionary conserved tetratrico peptide repeat protein pale yellow green7 is required for photosystem I accumulation in *Arabidopsis* and co-purifies with the complex. Plant Physiology **141**: 870-878

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Lectures

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Hein P (2005) A novel photosystem I mutant in *Arabidopsis*. Colloquium Endocytobiology. Bremerhaven.

Hein P (2006) *hcfb12-1*: an *Arabidopsis* mutant with defects in photosystem I. Colloquium Endocytobiology. Marburg.

Poster presentation

Stöckel J, Hein P, Bennewitz S, Oelmüller R (2004) Regulatorische Proteine für die Biogenese des Photosystems I in *Arabidopsis thaliana*. Molekularbiologie der Pflanzen. Dabringhausen

Curriculum vitae

Paul-Hendrik Hein

Dornburgerstr. 32

07743 Jena

paul.hein@uni-jena.de

Phone: 03641/511230

Date of birth: 29.04.1979

Place of birth: Halle

Education

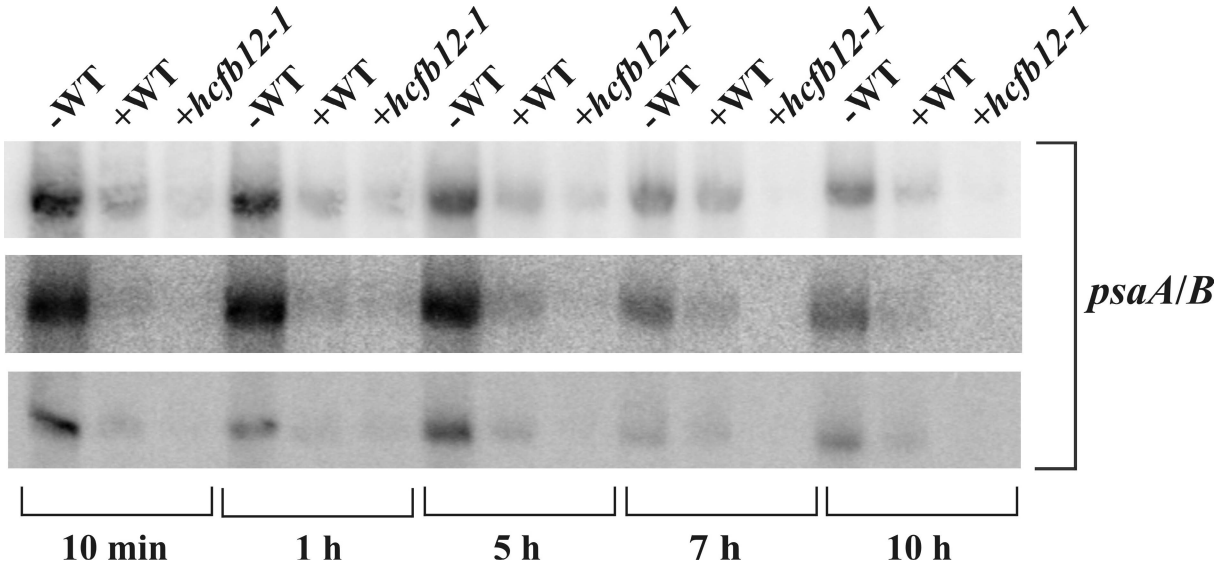
1985-1991	Elementary School, Beesenstedt
1991-1997	Grammar school, Wettin
1998-2003	Nutritional Science studies at the Friedrich-Schiller-University Jena
2001-2003	Certificate studies of Biology at the Friedrich-Schiller-University Jena
2003	Diploma thesis: Expressionsanalyse verschiedener Aminosäure- und Peptidtransporter in *Hordeum vulgare* L.

Work Experience

2002-2003	Student assistant at the Institut of Plant Genetics and Crop Plant Research, Gatersleben
since 2003	Research associate at the Institute of Plant Physiology, Friedrich-Schiller-University Jena

Addendum

Supplemental material page 47, Figure 9



psaA/*B* transcript stability for wild type and *hcfb12-1* mutant seedlings monitored over a period from 10 min to 10 hours using the transcription inhibitor actinomycin D. Seedlings were either treated with actinomycin D (+WT and +*hcfb12-1*) or water (-WT). Seedlings were incubated for 10 min, 1 h, 5 h, 7 h, 10 h prior total RNA isolation and *psaA*/*B* hybridization (cf. Northern analysis). 15 µg RNA were loaded per lane.

Ehrenwörtliche Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig und nur unter der Verwendung der angegebenen Hilfsmittel und Literatur angefertigt habe. Es wurde weder die Hilfe eines Promotionsberaters noch die Hilfe Dritter, nicht in dieser Arbeit erwähnten Personen, in Anspruch genommen. Diese Arbeit wurde weder in dieser noch in ähnlicher Form bei einer anderen Hochschule als Dissertation oder Prüfungsarbeit eingereicht.

Jena, 22.06.2007 ……..

Paul Hein